Studies on the mode of action of paraquat and diquat.

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STUDIES ON THE MODE OF ACTION OF PARAQUAT AND DIQUAT

Submitted by NICHOLAS HARRIS for
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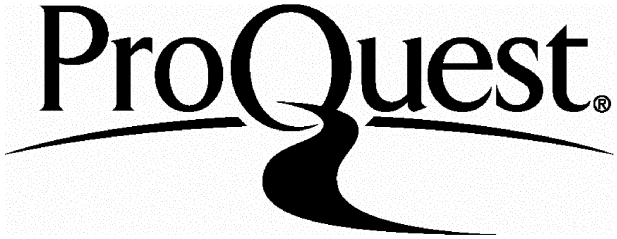
N. Harris



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ABBREVIATIONS

CMU	3-(4-chlorophenyl)-1,1-dimethyl urea
DCIP	dichlorophenol indophenol
DCMU	3-(3,4-dichlorophenyl)-1,1-dimethyl urea
NADP	nicotinamide-adenine dinucleotide phosphate
TRIS	2-amino-2-hydroxymethyl propane-1, 3-diol

A B S T R A C T

The mode of action of paraquat and diquat was studied by following some of the sequential events during the kill of flax cotyledon leaves by these two herbicides.

After floating the cotyledon leaves on the herbicide solutions the first effects noticed included an inhibition of photosynthetic carbon dioxide uptake. The production of toxic products by the interaction of the bipyridyls with photosynthetic electron transport was thought to cease before major breakdown of the cell components was completed. Hydrogen peroxide production was demonstrated with isolated chloroplasts incubated with paraquat and diquat. During the time that photosynthetic electron transport continued there was a major rise in the level of malondialdehyde, a product of lipid peroxidation, and an increase in cell membrane permeability. Both occurred at a time when electron micrographs showed disruption of the cell tonoplast and plasmalemma.

The bipyridyls are considered initiators of the kill process by indirectly effecting a loss of membrane integrity and a consequent loss of cellular organisation. The possible role of the vacuole acting as a lysosome is discussed with regard to the later breakdown of cell components.

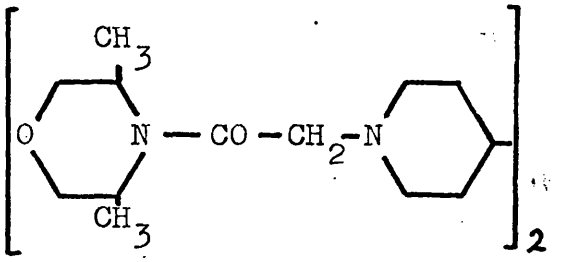
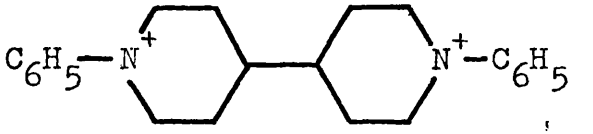
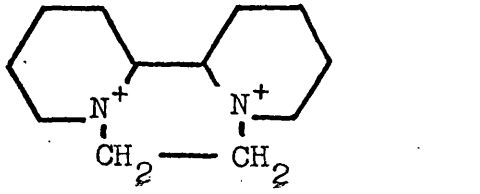
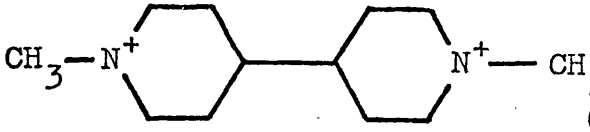
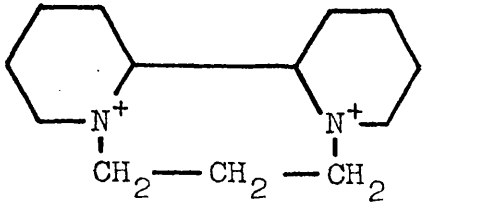
The interaction of paraquat and diquat, and other bipyridyls, benzyl viologen, morfamquat and triquat, with photosynthetic electron transport was investigated by studying the direct reduction of the bipyridyls and their subsequent reoxidation and also their inhibition of photosynthetic NADP reduction.

I N T R O D U C T I O N

The programme that led to the discovery of the herbicidal activity of the bipyridylium compounds was started in 1954. At that time it was known that among the surface active agents the quaternary ammonium compounds as a class were more phytotoxic than the anionic or non-ionic types. The first substance examined was cetyl trimethyl ammonium bromide, which desiccated young seedlings but required rather high rates of application to do so. During the screening of a range of quaternary ammonium salts it was discovered that 1,1'-ethylene-2,2'-dipyridylium dibromide (Diquat) had a high phytotoxic activity, 120 times greater than that of the first surface active agents tested. This work, and much of the subsequent research on the now widely used quaternary ammonium herbicides (Fig.1 shows structural formulae) was carried out at the Plant Protection Ltd. laboratories at Jealott's Hill, Bracknell.

Diquat was found to be an extremely quick acting herbicide, the kill being characterised by desiccation of the leaves and other non-woody green tissue. Under some conditions diquat was found to be translocated within the plant, but normally it gave top kill only (Brian, Homer, Stubbs and Jones, 1958). Tracer experiments had indicated that very little diquat was normally translocated to the roots. This result was in agreement with the finding that although quite extensive top kill of perennials could be obtained, rapid regeneration followed. Application to the soil produced no effects on the plants, a phenomenon found to be due to the adsorption of the highly polar herbicide on to the soil by an ion exchange mechanism. It is clear, however, that uptake by the plant is possible through the roots since addition of diquat to a culture solution results in a

Figure 1.

Morfamquat	1,1'-bis(3,5-dimethylmorpho-carbonylmethyl)-4,4'-dipyridylium dichloride.	
		E_o' (volts) (.305)
Benzyl viologen	1,1'-dibenzyl-4,4'-dipyridylium dichloride.	
		(.320)
Diquat	1,1'-ethylene-2,2'-dipyridylium dibromide.	
		(.348)
Paraquat	1,1'-dimethyl-4,4'-dipyridylium dichloride.	
		(.446)
Triquat	1,1'-trimethylene-2,2'-dipyridylium dibromide.	
		(.538)

rapid death of the plants (Brian, Homer, Stubbs and Jones, 1958).

Homer and Tomlinson (1959) and Homer, Mees and Tomlinson (1960) showed that the 2,2'-bipyridylium and also the 2,4'-bipyridylium and 4,4'-bipyridylium compounds were herbicidally active, whereas the 2,3'-bipyridylium and 3,3'-bipyridylium salts were inactive. It was shown that the compounds having some herbicidal activity similar to that of diquat, could be reduced to a free radical form by the addition of an electron, and conversely, compounds which could not be reduced in this way were not highly herbicidal.

An initial hypothesis put forward by Homer, Mees and Tomlinson (1960) was that for phytotoxic activity a coplanar molecule was required. However, this was not the only requirement for activity since two coplanar series, the 2,3'- and 3,3'-bipyridyls were inactive. It had long been known that the behaviour of 4,4'-bipyridylium on reduction was complex, the reaction taking place in two steps by way of an intensely coloured intermediate. Michaelis and Hill (1933) showed that the first step in the reduction involved the addition of one electron to the quaternary salt to form stable, water soluble free radicals. They examined four salts and found that the E'_o values varied with the nature of R associated with the bipyridyl, thus where R = methyl the E'_o =-446mV and where R = benzyl the E'_o = -320mV. These compounds have subsequently been used as redox indicators under the name of viologens, and are unique among known organic redox compounds in that the reductions in aqueous solution involves electron transfer and not hydrogen uptake. The first stage of the reduction of 1,1'-ethylene, 2,2'-bipyridylium is shown (Fig. 2A).

Figure 2A. Reduction of diquat to a free radical series.

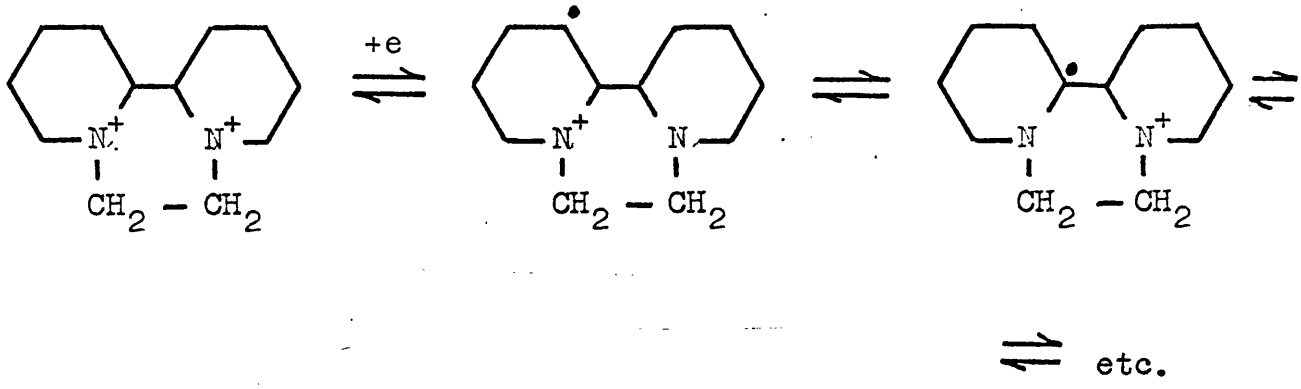
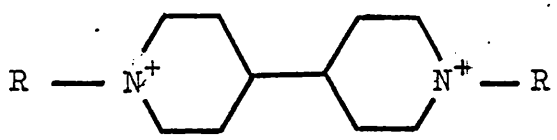
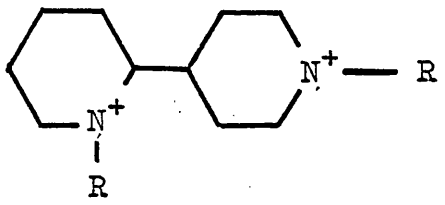


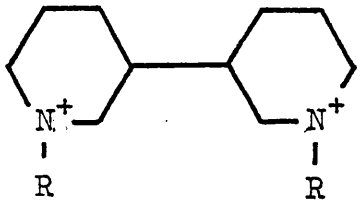
Figure 2B.



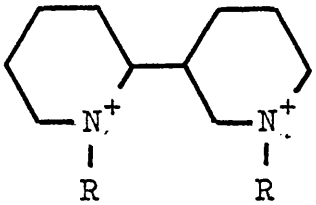
4,4'-bipyridyl



2,4'-bipyridyl



3,3'-bipyridyl



2,3'-bipyridyl

From the shape of the redox curve Michaelis and Hill suggested that the reaction involved only a one electron transfer and that a free radical was formed. It became apparent that the requirement of coplanarity was a necessary condition for the stability of the free radical (Homer and Tomlinson, 1959), since such stability depended essentially on the delocalization of the odd electron over the whole molecule.

Homer, Mees and Tomlinson (1960), in a revised hypothesis, suggested that phytotoxicity and reduction were related and that the former depended on the ability of the compounds to form stable free radicals. On this basis phytotoxicity was predicted and then demonstrated in the 2,4'-bipyridylium dimethiodide which Krumholtz (1951) had recorded as giving a purple colour with zinc dust. The activity, however, was found to be low and the redox potential more negative (-640 mV) than that of either diquat or paraquat (1,1'-dimethyl-4,4'-dipyridyl). It was consequently suggested that phytotoxicity was proportional to the amount of free radical produced. Based on the relationship :-

$$E = E_o + \frac{RT}{F} \cdot \log \frac{(ox)}{(red)}$$

Homer, Mees and Tomlinson (1960) calculated the proportion of free radical present in solutions of the various active compounds at various applied potentials and then related this to the minimum concentration of the chemical needed to kill i.e. the threshold concentration. They found that a theoretically applied potential of -380mV would give only a threefold variation in the calculated free radical concentration although the strengths of the solutions varied over 350 times. The value of -380mV was considered reasonable as it

was close to the value found for the reduction of NADP by chloroplasts (Tagawa and Arnon, 1962).

An explanation of phytotoxicity based on redox value alone is inadequate since the di-n-amyl quaternary salt of the 4,4'- series, whilst having a redox value similar to that of paraquat, has a threshold value ten times greater. Also, benzyl viologen, whilst having a redox value similar to that of diquat, has almost no herbicidal activity. It is suggested that in these cases physical limitations such as permeability may be interacting to modify the expected toxicity (Homer, Mees and Tomlinson, 1960).

Mees (1960) reported that light was essential for the herbicidal activity of diquat. Absorption and translocation in the dark was shown by the rapid death of treated plants when brought into the light. Death of broad bean leaf discs was found to be accompanied by the development of a black colouration, and it was reported that when these were floated on a solution of the herbicide they blackened at a rate proportional to the intensity of the incident light, up to values of at least 10,000 lux. Only green tissue was affected rapidly in the light, etiolated shoots failing to develop chlorophyll after treatment with diquat and thus only being killed slowly. Although light was found to be essential for rapid kill of green tissue by diquat, the compound was eventually lethal in the dark also.

Monuron, an inhibitor of photosynthetic electron transport (Wessels and van der Veen, 1956; Spikes, 1956), was found to inhibit the rate of kill by diquat in the light (Mees, 1960). As monuron

affected the activity rather than uptake it was concluded (Mees, 1960) that there was an interaction between diquat and photosynthetic electron transport. Potassium cyanide, which limited carbon dioxide uptake and respiration, was found to have no effect on the speed of kill in the light.

Cronshey (1961) suggested that the connection between photosynthetic electron transport and phytotoxicity could be that diquat was reduced to a free radical along with, or instead of, material normally reduced in the plant. Horowitz (1952) had found that there was an oxygen uptake when benzyl viologen was added to chloroplasts which were illuminated, thus giving some indirect evidence of reduction and reoxidation of the viologen in a photosynthetic reaction.

Both Homer, Mees and Tomlinson (1960) and Mees (1960) had concluded that reduction to the free radical was not enough for the rapid herbicidal action. It was suggested that free radical chain reactions might well involve the degradation of proteins and other large molecules of the protoplasm although there was no proof of this. Mees (1960) had quoted a communication from Brian drawing attention to the similarity between the effect of oxygen on the action of diquat and on the toxicity of ionizing radiation. In the latter case there is an induced radical formation and in the presence of oxygen it was believed that peroxide radicals were the toxic agents (Harman, 1963).

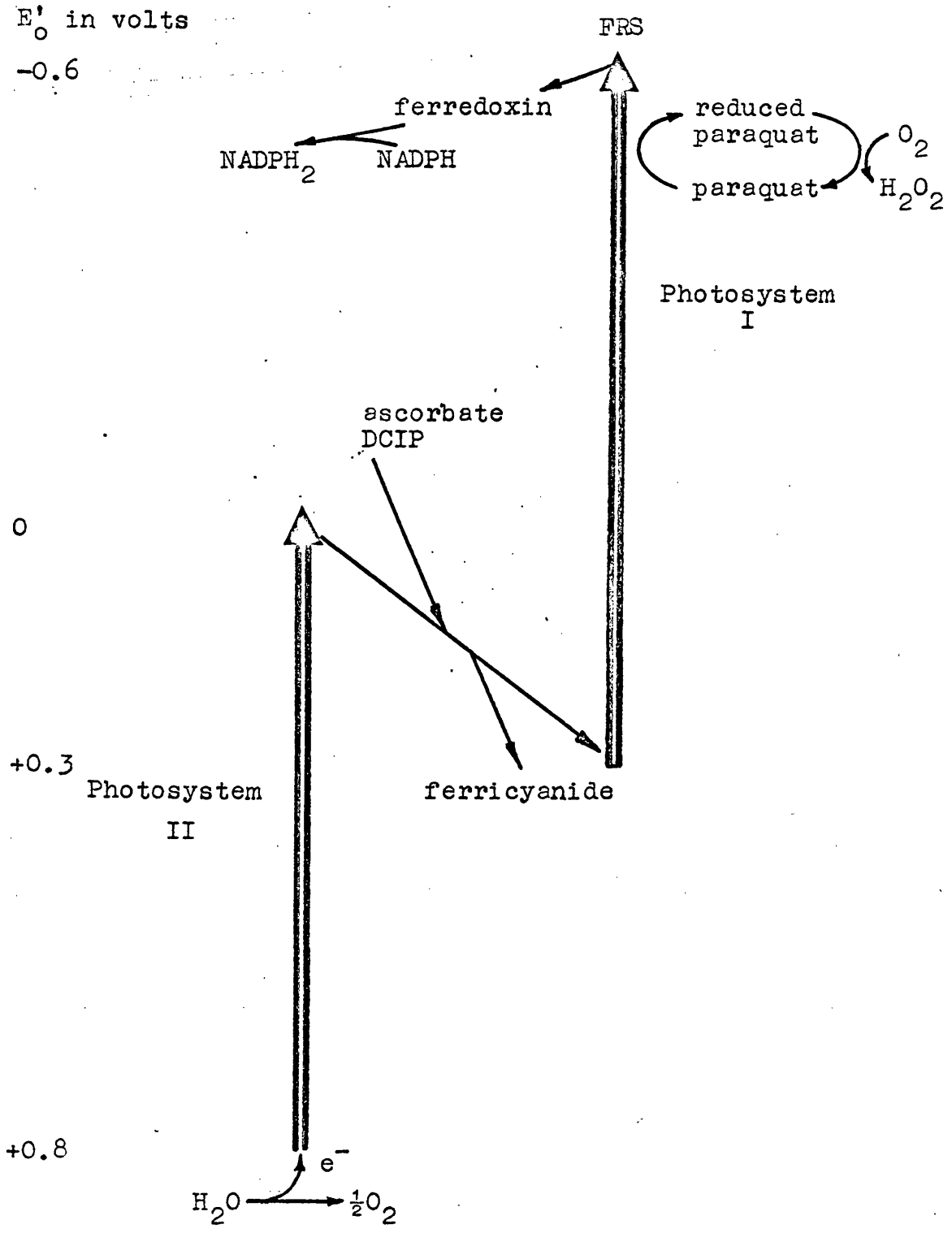
Some support for a hypothesis of linking diquat with hydrogen peroxide had been obtained by Baldwin (1960, unpublished

results quoted in Calderbank, 1964), who demonstrated hydrogen peroxide formation after the oxidation of chemically reduced diquat. Davenport (1960) had shown spectroscopically the formation of a metmyaglobin-peroxide complex by illuminating a crude chloroplast preparation containing diquat.

Calderbank and Crowdy (1962) concluded that the bipyridylium salts exerted their herbicidal effect through reduction and subsequent reoxidation, thus replacing or short-circuiting the normal redox systems in the plant. Kok and Hoch (1963), Arnon (1963), Whatley and Grant (1964) and Kok, Rurainski and Owens (1965) observed a limited reduction of paraquat by fresh chloroplasts under anaerobic conditions.

In 1965 Zweig, Shavit and Avron published the results of a series of experiments on the interaction of diquat with photosynthetic electron transport. They demonstrated non-cyclic photophosphorylation coupled to photoreduction of diquat with a P/2e ratio of about 1 which could be inhibited with DCMU. An observation that diquat competitively inhibited photophosphorylation catalysed by PMS suggested that diquat and PMS occupy the same site of interaction with the electron transfer pathway in chloroplasts. This, and other evidence such as the lack of inhibition by diquat of the photoreduction of ferricyanide and DCIP and the strong inhibition by diquat of the photoreduction of NADP, led Zweig, Shavit and Avron (1965) to propose the scheme shown in fig. 3. Other data supporting this conclusion included a lack of a ferredoxin requirement for the photoreduction of diquat under conditions when ferredoxin was an absolute requirement

Figure 3. The interaction of paraquat with the pathway of photosynthetic electron transport (after Zweig, Shavit and Avron, 1965).



for the photoreduction of NADP, the ability of illuminated chloroplasts to almost completely reduce diquat and the negative redox potential of diquat (Zweig, Shavit and Avron, 1965).

It was established by Funderburk and Lawrence (1964) and also by Slade (1965 and 1966) that paraquat was not metabolised by the plant. In solution paraquat was not broken down by sunlight but when adsorbed onto surfaces breakdown was facilitated.

Although the site of action had been pinpointed and the production of a free radical presumed, the sequence of events which lead to plant cell death was unknown and was the principal purpose of this investigation.

Osbourne (1968) drawing attention to the similarity between some herbicide actions and natural plant senescence, commented that the action of the bipyridyls, although showing some apparent similarities, differed in having a much more rapid effect than is observed during natural senescence. The studies detailed below of the kill of flax cotyledon leaves by the bipyridyls paraquat and diquat are compared with changes which occur during natural senescence in order to show both similarities and differences between the herbicide kill and senescence. Although no studies of the sequential events during kill by the bipyridyls had been reported, there were a number of papers on investigations of the course of senescence.

Shaw and Manocha (1965) followed chlorophyll, protein and nucleic acid breakdown in detached, senescing wheat leaves and correlated these changes with structural changes shown by electron microscopy. Similar studies with senescing cucumber cotyledon leaves

were reported by Lewington, Talbot and Simon (1967) and Butler (1967). Ikeda and Ueda (1964) had studied the structural changes in chloroplasts of senescing leaves of Elodea and found that as the thylakoids broke down large osmiophilic globules appeared in the stroma. Butler (1967), also, found the accumulation of osmiophilic globules a most conspicuous feature of chloroplast breakdown. The changes in mitochondrial structure in cucumber cotyledons were not as great as those found in other senescing tissues (Bain and Mercer, 1964; Shaw and Manocha, 1965). Varner (1961) suggested that senescence might be accompanied by a failure of the system responsible for mitochondrial repair. Any subsequent shortage of ATP would result in a loss of membrane integrity, a redistribution of substrates within the cell and therefore a loss of metabolic control (Bain and Mercer, 1964). Such a redistribution would occur following the rupture of the tonoplast, an event which was held by Shaw and Manocha (1965) to be a major contributing feature in the course of senescence. However, Butler (1967) found that in cucumber cotyledons cytoplasmic breakdown was well under way before the tonoplast disappeared, although he could not rule out the possibility of leakage across the membrane.

Eilam (1965) found that permeability changes were an early sign of senescence but suggested that these changes were independent of the respiratory system. Although loss of membrane integrity would account for structural changes in senescence this need not necessarily be concerned with a deficiency of respiratory control, and Eilam concluded that mitochondrial changes were probably a

consequence of senescence rather than a cause.

Barton (1966) suggested that the initial damage to the cell could be the direct result of the release of hydrolytic enzymes, and De Duve (1959) had proposed that these were located in lysosome like organelles. Evidence for the occurrence of lysosomes in plant tissue is conflicting. Some authors (Avers, 1961; Gahan, 1965) have described a particulate distribution of reaction products from histochemical tests for acid phosphatase (Bitensky, 1963). However Barton (1966) was unable to find any such particulate reaction in Phaseolus. Similarly, Poux (1962) working with wheat meristematic tissue did not find any such localisation. Poux did observe, however, that the acid phosphotase was present in young vacuoles in meristematic cells of the shoot apex, suggesting the possibility that hydrolytic enzymes might be released into the cell cytoplasm on rupture of the tonoplast. More recently Matile has been compiling evidence that the vacuole is, in effect, a lysosome. The vacuoles of root tip cells were shown to contain a considerable number of the acid hydrolytic enzymes (Matile, 1966). Matile and Moor (1968) concluded that the complement of hydrolytic enzymes contained in the cell sap, within the vacuole, catalysed the breakdown of cytoplasmic material released by invaginations of the tonoplast, such as observed by Poux (1963) and Brandes, Beutow, Bertini and Malkoff (1965). Subcellular organisation dependent on tonoplast integrity was considered of fundamental importance in the control of metabolic processes (Matile and Moor, 1968).

The results and discussion that follow are concerned with the sequential chemical, physiological and ultrastructural changes occurring when paraquat and diquat were applied to cotyledon leaves of flax (Linum usitatissimum). The results and discussion also include some studies of the interactions of these two bipyridyls and morfamquat, benzyl viologen and triquat with photosynthetic electron transport.

MATERIALS AND METHODS

1. Growth of plant material

Linum usitatissimum (flax) was grown on expanded mica (Vermiculite). The seeds were sown on waterlogged medium in a seed tray without drainage holes, and were germinated under high humidity by covering the tray with a sheet of glass. The germinating seedlings were kept under continuous illumination of 1,250 lux from 80 watt 'day glow' neon tubes. The temperature varied seasonally between 18 and 25°C. Plant material was taken for treatment 8 to 10 days after sowing. At this time the cotyledon leaves had fully expanded but the primary bud had not yet begun to expand. The peas (var. Meteor), used in some experiments as a source of chloroplasts, were grown in moist peat (with drainage). The seed trays were kept in the greenhouse under a 14 hour day at approximately 18°C. The pea seedlings were harvested for chloroplast preparations when about 3 inches high.

2. Treatment with herbicides

The flax cotyledon leaves were treated by floating on the appropriate solutions, with distilled water as a control. The cotyledon pairs were cut with a few mm of hypocotyl, and then carefully placed on the herbicide solution in 10 cm crystallising dishes. Care was taken to ensure that the upper surfaces of the cotyledon leaves were not wetted.

Preliminary experiments had shown that there were no apparent changes, during the first 36 hours, in the rate of kill with cotyledons floated on or sprayed with diquat or paraquat.

At later stages, however, differences appeared as the sprayed material desiccated thus limiting the breakdown processes. With the floated cotyledon leaves it was essential that the upper surfaces were not wetted; if this did occur there was a marked reduction in the rate of kill, probably due to limited oxygen (Mees, 1960).

Flax cotyledon leaves were treated with paraquat (10^{-4}M aqueous), diquat (10^{-4}M aqueous), benzyl viologen (10^{-4}M aqueous), morfamquat (10^{-4}M aqueous), triquat (10^{-4}M aqueous), CMU (10^{-3}M aqueous) and kinetin (50 ppm). Combinations of the treatments were also used.

Illumination was supplied by 80 watt fluorescent 'day glow' tubes to give 2,250 lux incident on the treated leaves. For reproducibility of any particular treatment constant temperature was found to be essential, and this was achieved by placing the crystallising dishes in a constant temperature water bath.

Pigment Analysis

3. Estimation of chlorophylls.

Chlorophyll levels were measured following the method of MacKinney (1941) as modified by Arnon (1949). In some experiments checks were made by estimating chlorophyll levels by the method of Vernon (1960). The cotyledon leaves were ground in 80% acetone : 20% water in a glass mortar under low white or green light. The suspension was centrifuged at approximately 500xg for 5 minutes. A colourless pellet indicated that all of the chlorophyll had been

extracted. The optical density of the solution was measured at 663 and 645nm and by substitution in the formulae below the concentration of chlorophyll was determined :-

total chlorophyll (mg/l) $= (20.2 \times OD_{645}) + (8.02 \times OD_{663})$

chlorophyll A (mg/l) $= (12.7 \times OD_{663}) - (2.69 \times OD_{645})$

chlorophyll B (mg/l) $= (22.9 \times OD_{645}) - (4.68 \times OD_{663})$

Final results were expressed as mgm chlorophyll per gm fresh weight. The chlorophyll levels of chloroplast preparations were obtained by pipetting 0.3ml of the chloroplast suspension into 9.7ml 80% acetone and after centrifugation of the membrane debris optical densities were determined as above.

4. Estimation of pheophytin and carotenoid pigments

These pigments were assayed using 80% acetone extracts. Pheophytin levels were measured by the method of Vernon (1960) and carotenoid levels by using the molar extinction coefficient at 480nm (Vernon, 1960).

5. Separation of pigments by chromatography

In order to verify the pattern of changes shown by determination of pigment levels spectrophotometrically the pigments were separated chromatographically. Cotyledons were ground in 100% acetone and the quantities adjusted to give a final solution of approximately 80% acetone 20% water. The pigments were transferred to petroleum ether (40-60^{o}C) and applied to Whatman No.1 paper (31.5 x 33.5 cm). The chromatograms were run in descending tanks

with petroleum ether : benzene : acetone at 4:1:0.5 as the mobile phase. All preparations were carried out under green light and the chromatograms were run in the dark. After development the chromatogram strips containing the pigments were cut away and the pigments eluted with 80% acetone. Alternative separation techniques employed Whatman No.4 paper with a mobile phase of petroleum ether (60-80^{o}C) with 1% n-propanol. Alternative pigment extraction with non-aqueous solvents used petroleum ether (60-80^{o}C) : acetone at 2:1 with the solution applied directly to the chromatogram for development.

Lipid analysis

6. Extraction of lipids

Lipids were extracted with either chloroform:methanol at 2:1 or following the method of Bligh and Dyer (1959). In the latter case 8 gm fresh weight of cotyledon leaves were ground with 20ml methanol and 10ml chloroform for 2 minutes in a Waring blender. A further 10ml chloroform were added and maceration continued for a further 30 seconds. 10ml water were then added and the macerate blended for 30 seconds. After filtration the chloroform layer was separated and dried with sodium sulphate. When required the volume of the extract was reduced in darkness at 20^{o}C under vacuum.

7. Thin layer chromatography of lipids

Thin layers of 'silica gel g', either BDH or Merck, were prepared from a slurry of 50gm in 90ml distilled water. The thickness

of the layer was varied depending on whether the plates were for analytical or preparative separations. The coated glass plates were routinely activated at 110°C for 20 minutes immediately prior to use. Glass capillaries were used for loading samples onto the surface of the silica layers, care being taken not to disturb the silica. Samples were loaded under low illumination and where necessary drying of the spots was assisted by blowing a gentle stream of nitrogen over the plate. Although there was little chance of oxidation of the lipids during the actual development of the chromatogram (Mahadevan quoted in Nichols, 1964), the chromatograms were developed in the dark.

The solvent systems used included :-

diethyl ether : petroleum ether : glacial acetic acid at 30:70:1
(Appelquist, Boynton, Stumpf and von Wettstein, 1968)

di-isobutyl ketone : acetic acid : water at 80:50:7
(Marinetti, Erbland and Stotz, 1958)

chloroform : methanol : acetic acid at 80:25:1
(Nichols, 1964).

The chromatograms were run in sealed tanks (25 x 25 x 7cm) which were lined with a filter paper wick to ensure a solvent saturated atmosphere.

When plates were used for qualitative analysis solvents were removed by air drying in the dark. For quantitative analysis plates were dried in vacuo, or with a stream of nitrogen.

Standards were prepared to assist in the identification of the components separated from the total lipid extracts. These

markers were prepared by extracting total lipds from flax cotyledon leaves and applying to silicic acid column in diethyl ether. The pigments, neutral lipids and free fatty acids and sterols were eluted with diethyl ether. Then the monogalactosyl diglycerides were eluted with 10% methanol in diethyl ether (V/V) and the digalactosyl diglycerides with 25% methanol in diethyl ether.

When the running positions of the particular components had been established, the presence or absence was checked using phosphomolybdic acid spray. The dried plates were sprayed with dilute phosphomolybdic acid in methanol and were dried at 105^{o}C for 15 minutes. This was found to be more convenient than using a 50% sulphuric acid spray and charring for 20 minutes at 220^{o}C (Nichols, 1964).

Iodine vapour was used during the identification of components to show compounds with unsaturation. The developed chromatograms were dried and placed in a dry chromatography tank which could be sealed, and an atmosphere of iodine was produced by dropping a few crystals of iodine onto a crucible of hot sand within the tank. Phospholipids were identified using a molybdate-perchloric acid spray (Skidmore and Entenman, 1962). Periodate - Schiff reagent was also used in the identification of the components of the chromatograms (Baddiley, Buchanan, Handschumaker and Prescott, 1956). The plates were sprayed with 0.2% aqueous sodium perchlorate, left for 15 minutes and then treated with SO_2 to reduce the excess periodate. They were then sprayed with Schiff's reagent and, after

a brief treatment with SO_2, left to develop. Lipids with glycol groups turned purple whereas the phospholipids turned yellow.

8. Analysis of glycolipids

The nature of the major lipid component, the glycolipid, which showed significant breakdown during the herbicide treatment, was determined by deacylation of the glycolipids and decomposition of the glycosides. Analysis of the glycolipids followed the method of Brundish, Shaw and Baddiley (1967). The glycolipids separated by preparative thin layer chromatography were eluted with chloroform : methanol at 1:1 v/v, and 2.5ml of this were added to 0.63ml 0.5M sodium methoxide in methanol (Marinetti, 1962). After the reaction had proceeded for 10 minutes, at room temperature, water (1.5ml) was added and the mixture was applied to a column (1ml) of Dowex 50 resin in the hydrogen ion form. Eluting with 5ml water removed the glycoside and then elution with 5ml chloroform released the organic phase of mixed fatty acids and methyl esters.

The glycoside was analysed by first hydrolysing for 2 hours with 2N HCl at 100^{o}C. The products were desalted (Baird & Tatlock 3ml chamber) and then applied to Whatman No.1 chromatography paper. After separation with a solvent system of butanol : pyridene : water (6:4:3) (Jeanes, Wise and Dimler, 1951) the galactose and glycerol were identified using the alkaline silver nitrate reagent of Trevelyan, Procter and Harrison,(1950), and also with periodate-Schiff reagent for a-glycol. The fatty acid methyl esters were

examined using gas-liquid partition chromatography. A column 5 feet in length and $\frac{1}{4}$ inch in diameter was used with EGSS-X coating at 15% concentration and a mesh of 100/120. The column temperature was set at 195°C and the detector at 200°C. The carrier gas used was nitrogen (50ml/minute), hydrogen (50ml/minute) and air (730ml/minute).

9. Estimation of malondialdehyde

An estimation of lipid breakdown was obtained by monitoring the amount of lipid peroxidation, as indicated by the level of malondialdehyde, during the course of the herbicide action. Malondialdehyde was assayed using the thiobarbituric acid method (Kohn and Liversedge, 1944; Patton and Kurtz, 1951). A known weight of cotyledon leaves were ground in distilled water and 3ml of this preparation were incubated with 5ml of 0.5% TBA in 20% TCA for 30 minutes at 95°C. After cooling and centrifugation to give a clear supernatant, the optical densities of this solution were measured at 532 and 600nm. The difference between these values gave a quantitative stoichiometric relationship with the amount of lipid peroxidation (Packer, 1967).

10. Estimation of carbohydrate level

Carbohydrate estimations were carried out using the phenol/sulphuric acid method (Dubois, Gilles, Hamilton, Rebers and Smith, 1956). 0.5ml of the aqueous sample were placed in an acid cleaned boiling tube with 0.05ml 80% phenol in aqueous solution. 5ml of concentrated analar sulphuric acid was added rapidly and

after cooling the optical density at 490nm was determined. The optical density difference between the sample and a distilled water control was compared with a standard graph of optical density change with μMoles galactose.

Galactose levels were also determined using a galactose dehydrogenase method (Rommel, 1968). 0.2ml of a sample solution was added to 3.00ml 0.2 M phosphate buffer at pH 7.5 and 0.10ml of a 13mM solution of NAD in a cuvette. After mixing the optical density was measured at 366nm, and then 0.02ml of a suspension of 5mg galactose dehydrogenase per ml was added. The cuvette was incubated at 20°C for 40 minutes and the optical density at 366 was measured. Galactose was calculated from

ΔE_{366} x 90.5 x dilution factor = mg % galactose in sample solution.

11. Estimation of protein levels

Soluble protein was determined by the method of Lowry, Roseburg, Farr and Randall (1951). Cotyledon leaves were ground in 0.1M TRIS chloride at pH 7.0 and a clear supernatant was produced by centrifugation. 0.5ml of the aqueous solution was added to 3ml alkaline copper sulphate solution and after 10 minutes 0.05ml of a solution of standardised Folin's reagent was added. The sample was left at room temperature for 30 minutes and the change in optical density at 750nm compared with a distilled water blank.

12. Separation of active protein bands by gel electrophoresis

The method used for separation of proteins by gel electrophoresis was based on that of Davis (1964).

Polyacrylamide gels were formed in glass tubes (internal bore of 5mm) previously cleaned and rinsed in 0.5% aq. solution of PhotoFlo (Kodak Ltd.) as described by Davis (1964). 1ml of 7.5% acrylamide solution was used for the separating gel which was allowed to polymerise before 0.2ml of the stacking gel was run onto the top. Both were prepared as described by Ornstein (1964). After polymerisation the tubes were placed in the electrophoresis apparatus which was constructed from two rectangular perspex tanks placed one above the other (Laycock, Thurman and Boulter, 1965). The tubes were mounted vertically, connecting each end with the reservoir buffer solution (0.1 M TRIS glycine buffer pH 8.3) in such a way that the lower solution was the anode, and the upper one the cathode. Hence proteins, as anions, separate as they move downwards through the separating gels after a preliminary concentration at the large pore: small pore interface. A suitable volume of cell extract containing 250μgm protein in a maximum of 0.05ml was prepared. This was done by homogenising cotyledon tissue in 0.1M TRIS methylamine buffer pH 8.0 containing, per 20ml, 170gm sucrose, 1gm ascorbate and 1gm cysteine hydrochloride (Stahmann, 1963). Bromophenol blue was added to the buffer in the top tank to indicate visibly the position of the moving front. Effective separation of protein was obtained by using a constant current of 2.5 amp per gel column supplied from a Vokam 400 volt power supply. Electrophoresis

carried out at room temperature, was continued until the marker dye had migrated to within 2mm of the base of the tube.

After electrophoresis the gels were removed from the tubes by gently loosening them from the glass with a hypodermic needle through which distilled water flowed.

13. Staining of protein bands

a) Protein. Total protein was detected by immersing the gels in a solution of 1% naphthalene black in 20% acetic acid for 1 hour. Excess dye was removed overnight by destaining with 7% acetic acid.

b) Catalases. To detect catalase activity the gels were treated with 0.2M aqueous catechol for 30 minutes, washed with distilled water and soaked in 0.3% H_2O_2 for 5 minutes until brown bands developed on a white background.

c) Recording of results. Gels stained for protein and isoenzyme activity were placed in small glass, polythene capped tubes. Densitometer tracings of the gels were obtained by scanning the gels with a Joyce-Loebl Chromoscan using the appropriate wedges, filters and slit sizes. As the experiments were comparative, the same conditions were used for scanning the control and treated extracts. Although the protein gave a stable colour reaction on the gel, it was necessary to scan for catalase activity within 5-10 minutes of the staining reactions.

14. Estimation of membrane damage

The rate of efflux of potassium from slices of leaf was used as an indicator of the degree of membrane damage (Eilam, 1965). 1 mm slices of leaf were washed in distilled water to remove potassium of the cells damaged in the sectioning of the tissue. The slices were then incubated in distilled water at 20°C and aerated. Aliquots of the water were taken at 10, 20 and 30 minutes and the potassium levels of these samples were measured using flame photometry. The residual potassium within the leaf was released by boiling for 10 minutes, after which the volume of water was made up to a known level and the concentration of potassium determined.

The effect of diquat and paraquat on membrane permeability was investigated by measuring both the efflux rate from pre-treated leaves, and also by measuring the rate of efflux of potassium from slices incubated in aerated herbicide solutions under illumination.

Comparison of the rates of potassium ion efflux from herbicide treated and control leaf slices gave an indication of the degree of membrane damage, in particular damage of the tonoplast which normally retains a high proportion of the cell potassium ions within the vacuole (Diamond and Solomon, 1959).

Examination of structural changes in cotyledon leaves treated with diquat and paraquat

15. Light microscopy

Cotyledon leaves treated with paraquat and diquat were examined and compared with control tissue using a Zeiss Standard WL microscope with camera attachment. Micrographs were produced using Kodak Panotomic X film.

The cotyledon leaves were examined after either sectioning or teasing apart in TRIS chloride (0.3M at pH 7.0).

16. Electron microscopy

Following treatment with diquat or paraquat the cotyledon leaves were immediately cut into small pieces, approximately $1mm^3$, in fixative. The fixatives used were 1% $KMnO_4$ with a fixation time of 30 minutes (Luft, 1956), or a double fixation method involving glutaraldehyde and osmium tetroxide (Pease, 1964),where a primary fixation with 3% glutaraldehyde in phosphate buffer (at pH 7.0) for 20 minutes (Sabatini, Bensch and Barrnett, 1963, 1964) was followed by a post-fixation with osmium tetroxide (Palade, 1952) using a 1% solution in phosphate buffer for 1 hour at $4^{o}C$. Between the fixations the material was thoroughly washed using phosphate buffer at pH 7.0. Mohr and Cocking (1967) recommended a similar double fixation technique for use with vacuolated, senescent or damaged plant tissue. Following fixation with potassium permanganate the material was washed with distilled water to remove excess fixative,

while excess osmium tetroxide was removed by washing with phosphate buffer.

After washing the material was dehydrated in an alcohol series and brought to propylene oxide. Following two rinses with propylene oxide (Luft, 1961) the material was left for a few hours in an equal volume mixture of propylene oxide and araldite. The mixture was replaced with araldite alone and the material was left overnight. The pieces of cotyledon leaf were finally transferred to fresh araldite and placed either in gelatin capsules or plastic dishes ($1\frac{1}{2}$ in diameter x $\frac{1}{8}$ in), with polymerisation at 60°C for 36 to 48 hours. The araldite used was prepared from 100ml araldite, 100ml hardener, 3ml accelerator and 3ml plasticiser (dibutyl phthalate).

Sections with a silver to silver-grey interference colour (approximately 600A thick, Peachey, 1958) were cut using an LKB microtome with glass knives. The sections were mounted on Formvar coated 200 mesh copper grids.

The sections were stained on the grids using alkaline lead citrate (Reynolds, 1963) for 5 to 20 minutes. The glutaraldehyde / osmium tetroxide fixed material was first stained with uranyl acetate (Watson, 1958) and then with lead citrate (Pease, 1964).

The sections were examined using a Zeiss EM 9 electron microscope.

17. Preparation and examination of protoplasts

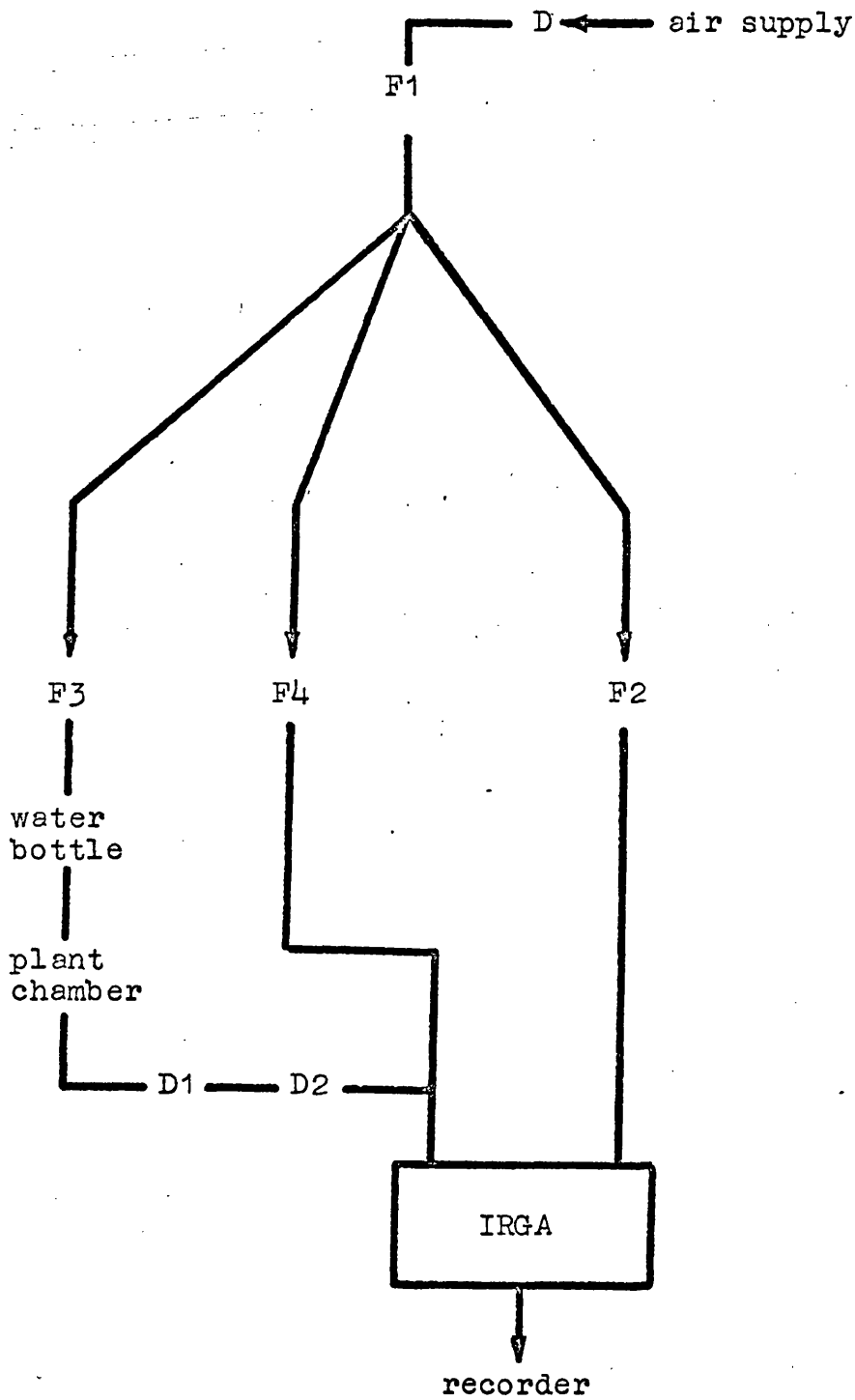
Protoplasts were prepared from flax cotyledon leaves and also from pea leaves. The leaves were cut into 1mm strips and treated with a crude cellulase preparation (kindly supplied by D.Wright, Bath University of Technology), obtained from a culture filtrate of Cephalosporium sp. grown on native cotton. Although the activity of the preparation was not assayed it was thought to contain cellulase and pectinase activity. The enzyme preparation was made up in 0.1M phosphate buffer at pH 5.6 with 0.25M sucrose to such a strength that the cellulose cell wall was removed within $1\frac{1}{2}$ to 2 hours. The flax cotyledon leaves required considerably more cellulase than the pea leaf strips.

Protoplasts were kept in sucrose / phosphate buffer, during which time they could be treated with diquat and paraquat, and were examined using a Zeiss Standard WL microscope with camera attachment.

18. Measurement of carbon dioxide changes

An infra red gas analyser (Grubb Parsons Ltd.) was used to measure changes in the carbon dioxide content of the air passing over plant material in a perspex container (100 x 14 x 6mm). A cylinder of high purity compressed air provided the gas stream (see Fig. 4) which passed through a combined pressure regulator and flow meter (F_1) and was divided into two. One stream supplied the air for the reference tube of the gas analyser after passing through F_2 whilst the other supplied the sample chamber. The sample stream, after passing through the flow-meter F_3 and water bottle at

Figure 4. Diagram of the air stream supplying an infra-red gas analyser (IRGA) recording the carbon dioxide changes of plant material contained within the plant chamber.



constant temperature, entered the plant chamber where its carbon dioxide content was modified by up to 550mg cotyledon leaf material. Both gas streams passed through drying towers (D_1 and D_2) of anhydrous calcium chloride before entering the analyser. A facility was provided whereby the plant chamber could be bypassed via a flowmeter (F_4) thereby enabling the infra-red absorption in both the reference and the sample tube to be equalised. The plant chamber was illuminated by a high intensity lamp, the heat evolved being absorped by a screen of running water between the lamp and the sample chamber. The flow rate of the air stream in both the reference and sample tubes was adjusted to 400ml per min. The results were expressed as μM carbon dioxide exchanged per gm fresh weight of cotyledon leaf per hour.

Reactions of isolated chloroplasts

19. Isolation of chloroplasts

Chloroplasts were isolated from pea plants using a method similar to that of Hill and Walker (1959). Pea plants were harvested when approximately 3 in high, washed with water, and then ground in an isotonic media in a mortar previously chilled to $0^{o}C$. The grinding media used contained 0.1M TRIS chloride buffer at pH 7.8, 0.35M sodium chloride and 0.03M magnesium chloride. After grinding the coarse debris was filtered using eight layers of prewashed muslin, and the supernatant was

then centrifuged at 500 x g for 3 minutes at 5°C. After centrifugation the supernatant was transferred to another cooled centrifuge tube and spun at 5,000 x g for 5 minutes at 5°C. The supernatant was discarded and the pellet resuspended in fresh cold grinding medium and recentrifuged as before at 5,000 xg. The pellet was then resuspended in a few mls 'breaking medium' (breaking medium = 1/10 dilution of the grinding medium). To ensure the production of a uniform suspension a pre-cooled glass Potter-Elvehjem homogeniser was used and the homogenate was filtered through glass wool into a tube held in an ice bath. A chloroplast suspension produced in this way was found to give samples which retained uniform activity for at least 3 hours.

The preparation of chloropast suspensions from flax required a modified procedure to ensure reproducibility in activity. Flax cotyledons were ground in a cold mortar as above and after filtering through muslin were centrifuged at 8,000 xg for 1 min. The forces exerted using an MSE 18 cooled centrifuge in taking an 8x50 fixed angle head to 8,000 xg and then slowing rapidly after 1 min were sufficient to sediment a pellet of flax chloroplasts. The pellet was suspended in 'breaking medium' using a cooled homogeniser as before. After filtering through glass wool the suspension was used immediately to achieve maximum rates in Hill activity and ascorbate photo-oxidation. Using this technique, Hill activity could be assayed with 8 min from harvesting.

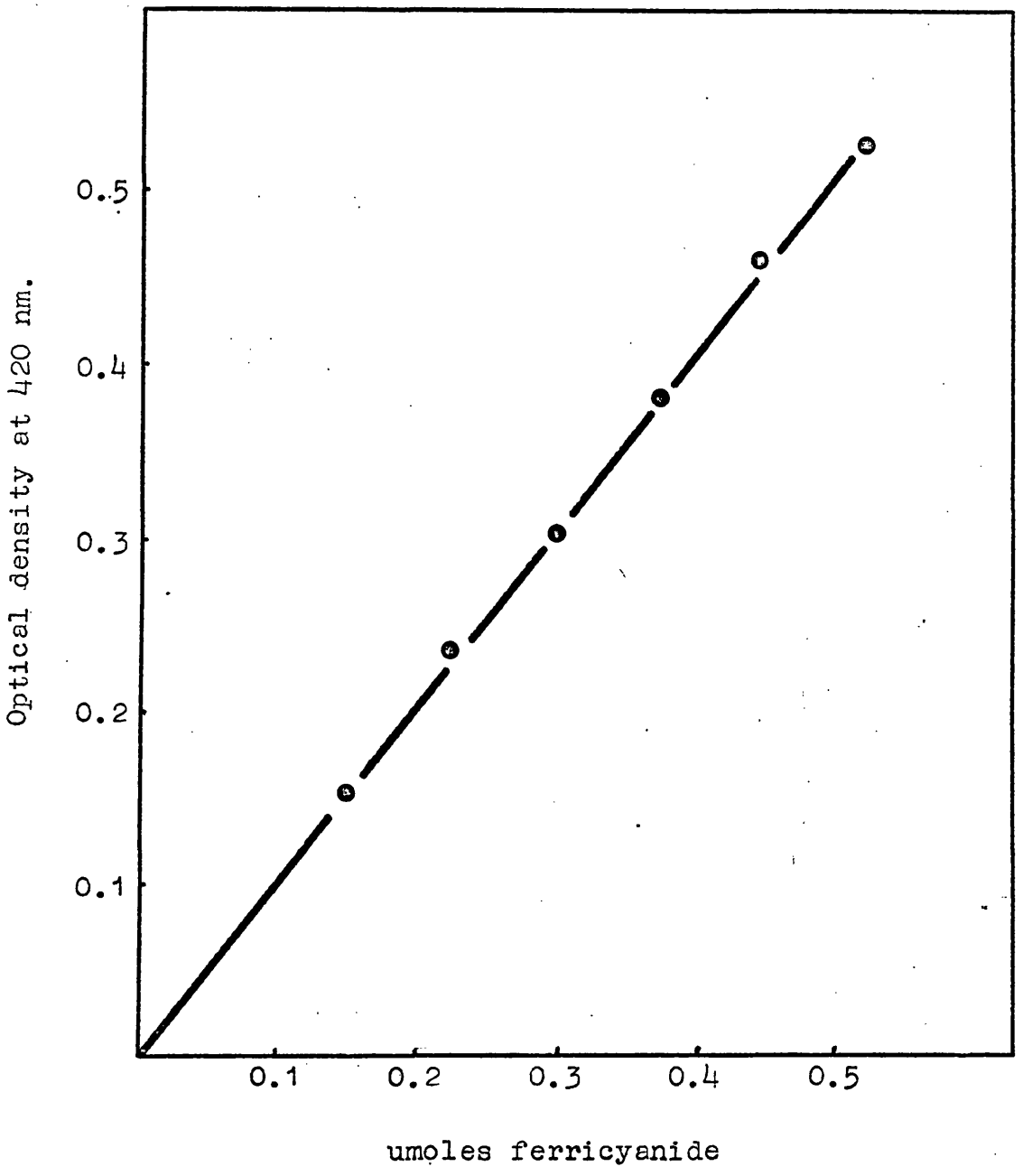
20. Quantitative estimation of Photosystem II activity (Hill reaction)

Ferricyanide reduction in the Hill reaction was determined spectrophotometrically at 420nm (Jagendorf and Marguilles, 1960) using a Unicam Sp 500. The reactions were carried out in 1cm silica cuvettes, containing usually 0.1 to 0.3ml chloroplast suspension (approximately 20 µg chlorophyll), 0.3ml TRIS buffer (0.3M pH7.8), 0.1ml 10^{-2}M potassium ferricyanide and the volume made to 3ml with distilled water. The contents of the cuvettes were mixed by shaking and were then illuminated for 2 minute periods and the optical density changes measured after each illumination. Saturating white light of approximately 50 k lux was used for illumination with a dilute copper sulphate solution interposed between the lamp and cuvettes to absorb heat. The rate of ferricyanide reduction was determined by comparing the optical density changes against a standard graph of optical density / ferricyanide concentration (Fig. 5). Hill activity is expressed as µmoles ferricyanide reduced /mgm chlorophyll / hour.

21. Quantitative estimation of Photosyntem I activity (ascorbate photo-oxidation)

Oxygen exchange during the oxidation of ascorbate by Photosystem I was measured both manometrically and with an oxygen electrode. The isolated Photosystem I activity was measured by substituting ascorbate for water in a Mehler type system (Mehler, 1951a,b). The variation, as described by

Figure 5. Calibration graph for the spectrophotometric estimation of ferricyanide.



Davenport and Dodge (1969), involved the inhibition of Photosystem II with CMU and the replacement of water as a source of electrons by ascorbate. With normally prepared chloroplast suspensions a link is needed between ascorbate and Photosystem I, dichlorophenol-indophenol (DCIP) frequently being used as a couple (Vernon and Zaugg, 1960). If the chloroplasts are damaged to a degree by, for example, heating, sonication or detergent treatment, the necessity for this couple is lost (Davenport and Dodge, 1969).

Manometric measurements were carried out using a Braun V85 Warburg apparatus with illumination through the perspex water bath giving an intensity of 11.8 klux at flask level in the bath. The reaction flasks contained 0.3ml TRIS (0.3M at pH 8.0), 0.2ml ascorbate (0.2M), 0.1ml CMU (10^{-3}M in 50% ethanol), 0.1ml paraquat (10^{-4}M), 0.2ml DCIP (10^{-3}M) when included, and chloroplast suspension varying between 0.3ml and 0.5ml containing approximately 20 µg chlorophyll. The flask contents were made up to 3ml and the reaction rates were measured, after equilibration, at 25°C. Changes in volume were measured at 5 minute intervals and were converted to µl oxygen uptake by the standard formulae (Umbreight, Burris and Stauffer, 1964).

Photosystem I activity was also measured using a Clark type oxygen electrode. The apparatus used (from Rank Bros., Bottisham, Cambs.) included a cuvette surrounded by a transparent constant temperature jacket, thus enabling the cuvette contents

to be illuminated. The chamber contents were mixed continuously by a glass covered magnetic 'flea' which was activated by a magnetic stirrer motor beneath the chamber, and the changes in oxygen tension were recorded using a Smith's Industries Servoscribe recorder linked to the electrode.

The oxygen electrode was used to determine the DCIP requirement of chloroplast preparations from diquat and paraquat treated cotyledon leaves, and also to determine the quantitative oxygen uptake mediated by increasing concentrations of bipyridyls with fresh pea chloroplast suspensions.

In the former series of experiments chloroplast suspensions from the variously treated flax cotyledon leaves were incubated with ascorbate (0.2ml 0.2M), TRIS (0.4ml 0.3M), CMU (0.1ml 10^{-3}M), paraquat (0.1ml 10^{-4}M) and distilled water to 3.9ml. After equilibration in the dark to determine any slow auto-oxidation of the ascorbate (Orr, 1966) the rate of oxygen uptake was determined with the suspension illuminated with saturating white light. DCIP (0.1ml 10^{-3}M) was added and any change in the rate of oxygen uptake noted.

To determine the quantitative changes in rate of oxygen uptake with changing concentration of bipyridyl, washed pea chloroplast suspensions were used. The chamber included chloroplast suspension (0.3ml containing approximately 30 μg chlorophyll), TRIS (0.4ml 0.3M pH 7.8), CMU (0.1ml 10^{-3}M),

DCIP (0.1ml 10^{-3}M), ascorbate (0.2ml 0.2M) and water to 3.95 ml. The bipyridyls diquat, paraquat, triquat, morfamquat and benzyl viologen were added, after determining the initial rate of oxygen uptake in the light, by injecting 0.01ml of concentrations increasing from 10^{-6}M to 10^{-1}M, with the change in rate being noted after each addition.

The oxygen electrode was also used to show some evidence of the production of hydrogen peroxide during the interaction of the bipyridyls with photosynthetic electron transport. In these experiments heated chloroplasts (55°C for 10 minutes), having no Photosystem II activity and no requirement for DCIP (Davenport and Dodge, 1969), were used to mediate electron flow from ascorbate. Catalase activity associated with the chloroplast was inactivated during the heating. Chloroplasts, TRIS (0.4ml 0.3M), ascorbate (0.2ml 0.2M) and bipyridyl (0.1ml 10^{-4}M) were incubated in the electrode chamber in a total volume of 3.8ml (made up with distilled water) and illuminated. Catalase (0.1ml 1mg/ml was added in the dark and after equilibration the suspension was reilluminated and ethanol (0.1ml 50%) was added. Changes in the rate of oxygen uptake were recorded as before.

22. Preparation of ferredoxin

Ferredoxin was prepared by a method similar to that of Tagawa and Arnon (1962). Parsley (Petroselinum sativum) was used as a source, the petioles being removed from the washed plant material and the laminae frozen. These were ground to a powder in a chilled mincing machine and mixed with 0.05M TRIS-chloride buffer pH 8.0. The volume of the buffer added was in the ratio of 800ml buffer per kilogram frozen leaves.

The mixture was allowed to thaw completely and was then filtered through four layers of butter muslin. To the filtrate was added 50% (V/V) cold acetone (-20°C(with stirring, and left at 5°C for 15 mins. After centrifugation at 1000 xg for 15 mins at 0°C, the acetone concentration was increased to 75% (V/V). The resulting precipitate was recovered by filtration through a pad of celite supported on filter paper, and then washed with cold acetone. The celite was powdered after the acetone had completely evaporated and resuspended in a minimum volume of 0.05M TRIS chloride buffer pH 7.3. The celite was removed by filtration to produce a clear brown liquid containing the ferredoxin.

The protein solution was dialysed overnight in a cellophane dialysis bag against 0.005M TRIS chloride pH 7.3 at 5°C. The precipitate which formed during dialysis was removed by centrifugation at 6000 xg for 20 minutes at 0°C, and the supernatant fractioned on Whatman DE 11 cellulose powder. A column (6 x 1cm) was prepared from a slurry of the cellulose

powder in distilled water. It was washed with water several times and then equilibrated with 0.005M TRIS chloride pH 7.3. The dialysed preparation was poured onto the column and first eluted with the equilibrating buffer until the flavone had been removed. Elution of the column with 0.2M sodium chloride in 0.005M TRIS chloride pH 7.3 yielded the fraction containing the flavoprotein and plastocyanin. The ferredoxin was recovered by elution with 0.8M sodium chloride in 0.005M TRIS chloride pH 7.3, and was stored at -20^{o}C until required. For the purposes of this investigation it was not purified any further.

23. Estimation of photosynthetic electron transport to NADP reduction

The photosynthetic reduction of NADP was measured spectrophotometrically in 3ml cuvettes with 1cm light path, at 340nm. The method of San Pietro and Lang (1958) was followed, with water as the reductant and ferredoxin as an intermediate. NADP reduction could be calculated,as a change in optical density of 0.1 is equivalent to 0.048 uM NADP (Colwick, Kaplan and Ciotti, 1951). The reaction mixture contained pea chloroplast suspension with TRIS chloride (0.3ml 0.3M pH 7.8), 0.5 uM NADP, 0.1ml of a ferredoxin preparation (approximately 1.8 mg protein) and water to a total volume of 3ml.

NADP reduction was also followed with ascorbate as the reductant. In these cases ascorbate (0.2ml 0.2M),

DCIP (0.2ml 10^{-3}M) and CMU (0.1ml 10^{-3}M) were included in the reaction cuvettes. In all the experiments the reaction blank excluded ferredoxin.

24. Estimation of the quantitative reduction of some bipyridyls

The reduction of the bipyridyls was carried out in Thunberg adapted cuvettes with ascorbate as the reductant. Reduction of the bipyridyls was investigated at the following wavelengths :-

benzyl viologen....545nm	paraquat....600nm
diquat.....380nm	
morfamquat.....595nm	triquat.....420nm

with the reduction quantified by reference to standard graphs produced by reducing known quantities of the bipyridyls with sodium dithionite. The 1 cm cuvettes were flushed with nitrogen and their contents then kept under vacuum. The cuvettes contained a standard amount of chloroplast suspension (approximately 30 ug chlorophyll) which had previously been heated at 55^{o}C for 10 minutes, TRIS (0.3ml 0.3M), the bipyridyl (0.1ml 10^{-2}M), ascorbate (0.2ml 0.2M) in all but the reaction blank and distilled water to 3ml. After 2 minutes illumination with saturating white light the optical density change was noted. To check that the degree of reduction was not masked by any re-oxidation, the cuvettes were monitored again after a further minute incubation in the dark.

25. Determination of catalase activity

Flax cotyledon leaves were macerated in 0.1M phasphate buffer at pH 7.0 using a glass homogeniser which had previously been cooled by standing in ice. 1 ml of the homogenate was transferred to 10ml of 0.01M buffer with H_2O_2 (0.15ml 6% H_2O_2 in 100ml 0.01M phosphate buffer at pH 7.0) at 0^oC. At 2 minute intervals 2ml samples were withdrawn and added to 0.5ml $MnSO_4$ in sulphuric acid (0.466 gm per 100ml 2½% sulphuric acid). The samples were titrated against 10^{-3}M potassium permanganate, the end point being when there was a slight pink colouration. This method of determining catalase activity by back titration of unoxidised manganous sulphate was described by Maehly and Chance (1954).

In this investigation the results are only comparative, the titration values have not been converted to any of the various catalase activity units.

R E S U L T S

1. Changes in pigment levels

A major change occurring after the treatment of flax cotyledon leaves with diquat and paraquat was a change in colour (see Plate 1). The normal 'fresh' green colour was gradually replaced by a duller, slightly darker green and this in turn was lost as the leaves turned brown. If the cotyledon leaves were sprayed with the herbicides desiccation followed and this appeared to inhibit the later stages of colour change. Material treated in this way did not always reach the extent of browning which occurred when the cotyledon leaves were floated on the solutions of the herbicides. In the latter case the cotyledon leaves did not desiccate, although there was a loss of turgor and a decrease in fresh weight (Fig.6 and dry weight (Fig.7).

The results given below refer, unless stated otherwise, to changes in cotyledon leaves which were floated on solutions of the herbicides.

Figure 8 shows the change in chlorophyll a and chlorophyll b levels in cotyledon leaves floated on paraquat under continuous illumination. The same pattern of change was obtained with diquat treatment. In order to compare various treatments, the figures given below have been adjusted so that the chlorophyll levels were based on the initial fresh weights of the experiments. The adjustments were made from the decrease in fresh weight compared with the initial fresh weight as shown in Figure 6.

Plate 1.

control

60 hours paraquat

90 hours paraquat

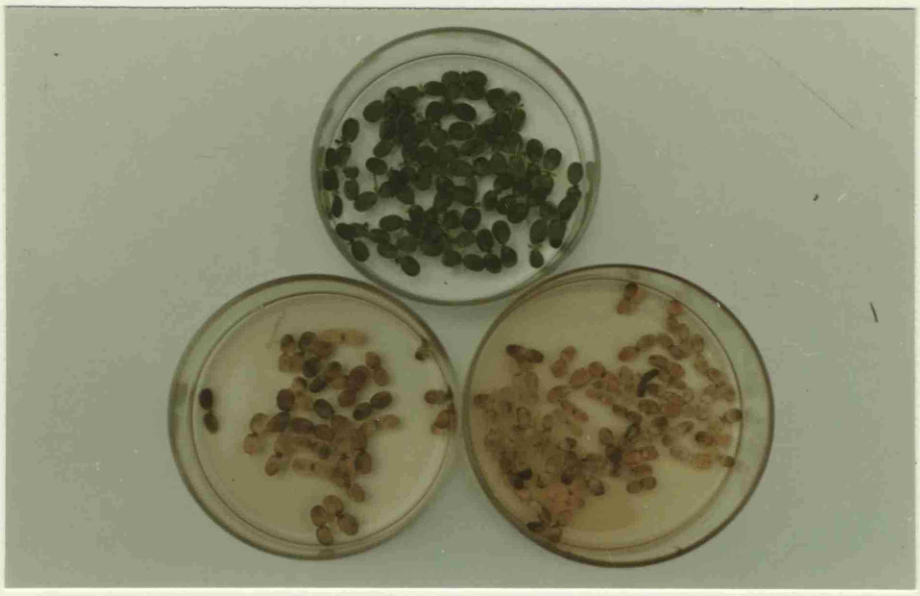


Figure 6. The effect of paraquat on the fresh weight of flax cotyledon leaves.

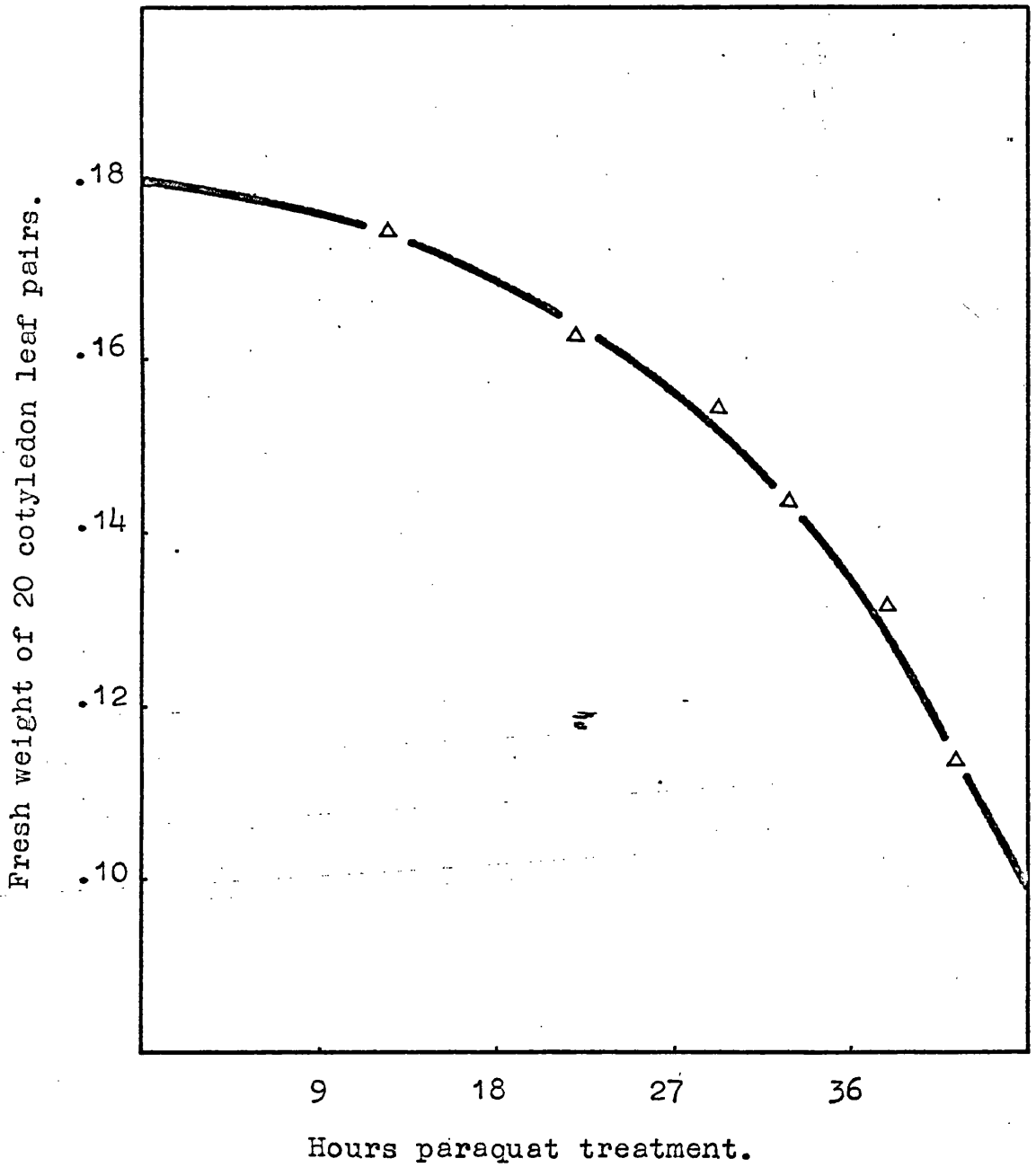
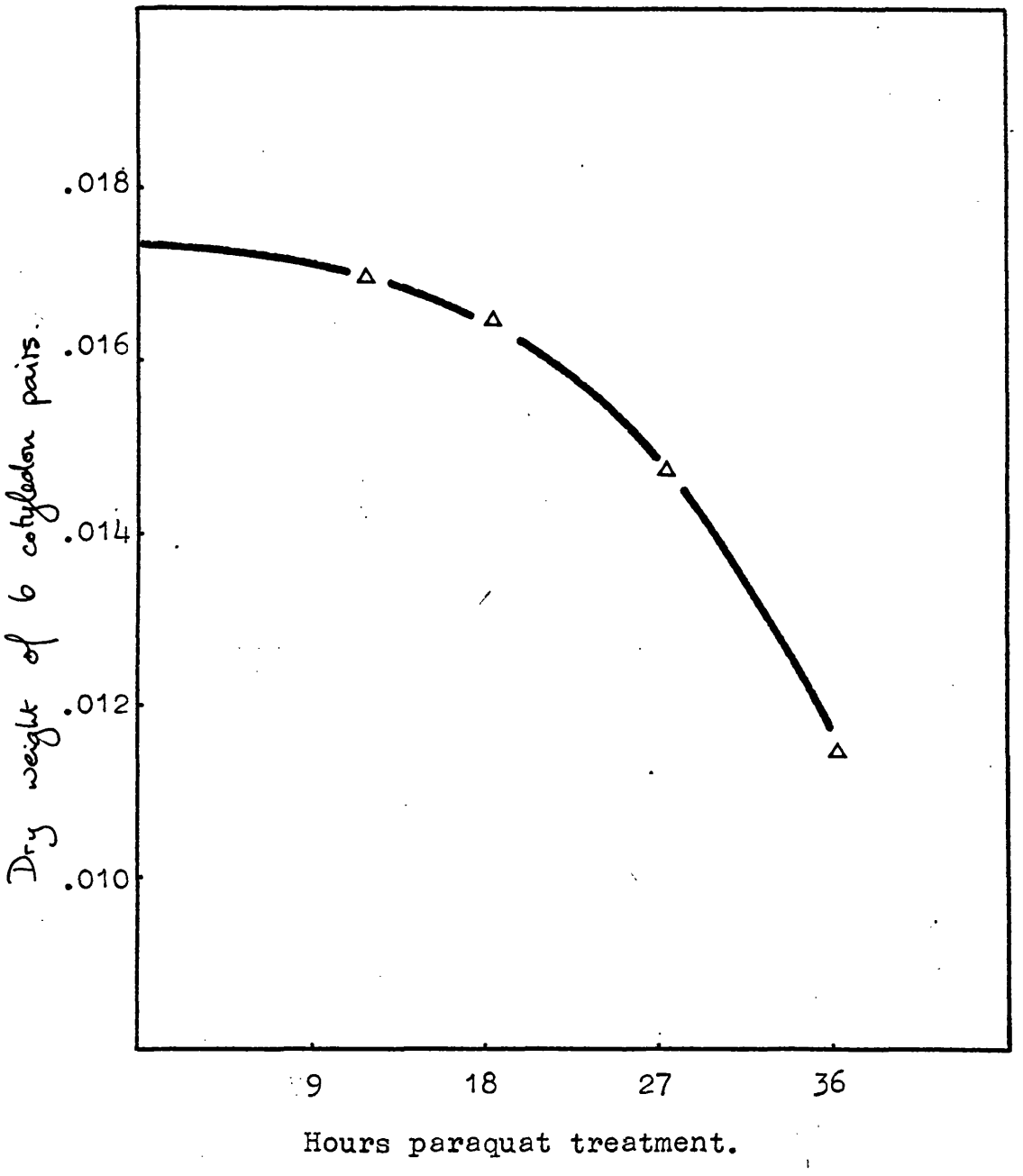


Figure 7. The effect of paraquat on the dry weight of flax cotyledon leaves.



The change in the chlorophyll a / chlorophyll b ratio in both natural senescence and during the kill by paraquat and diquat is shown in Figures 9 and 10. The changes in chlorophyll levels during the herbicide kill under varying periods of illumination and darkness are shown in Figure 11. It is significant that there was a reduction in the rate of breakdown in the dark after periods of illumination.

The effects of a retardant of senescence, kinetin, the photosynthetic inhibitor, CMU, and the general enzymic inhibitor, potassium cyanide, on the rate of chlorophyll breakdown in cotyledon leaves also treated with paraquat are shown in Figure 12. These results are of cotyledon leaves which were kept under continuous illumination.

The results indicate that there were considerable changes in the levels of the major pigments during the action of the bipyridyls diquat and paraquat on green leaf material. The effects of the photosynthetic inhibitor CMU appeared to be similar to those reported by Mees (1960) in inhibiting the loss of chlorophyll. The apparent lack of effect of kinetin might indicate a difference in the mechanism of the mode of action of diquat and paraquat as compared with the course of natural senescence, although the overall pattern of change in pigment levels was similar except in the rate of change. The inhibitor potassium cyanide appeared to have no significant effect on the rate of breakdown, indicating that respiratory processes do not play a major role.

Figure 8. The effect of paraquat on chlorophyll levels of flax cotyledon leaves.

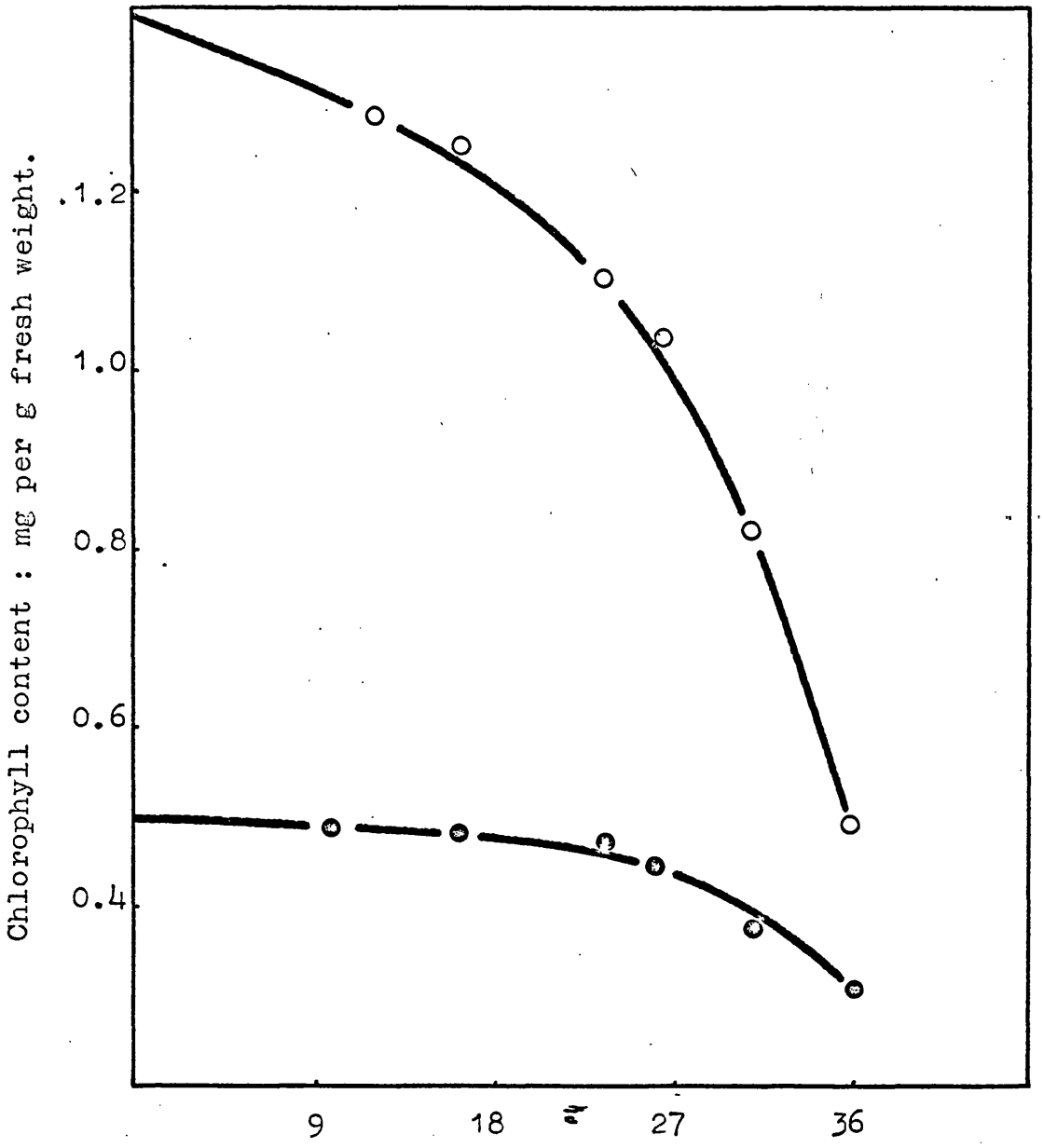
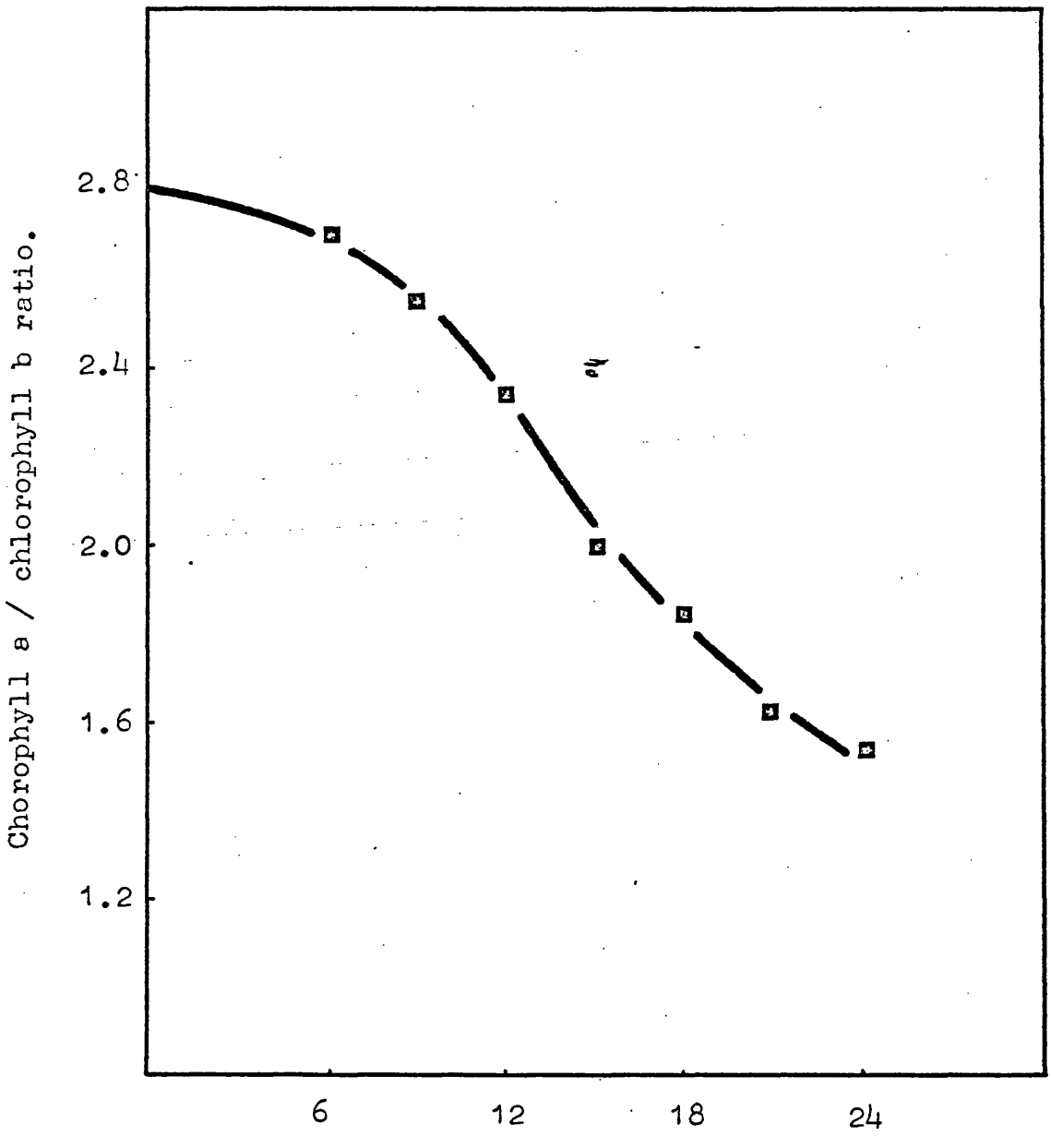


Figure 9. Chorophyll a / chlorophyll b ratio in senescing flax cotyledon leaves.



Days	7	11	15	20
chlorophyll a mg per g fr. wt.	1.30	1.15	0.86	0.51
chlorophyll b mg per g fr. wt.	0.50	0.48	0.45	0.30

Figure 10. The effect of the bipyridyls on the chlorophyll a / chlorophyll b ratio of flax cotyledons.

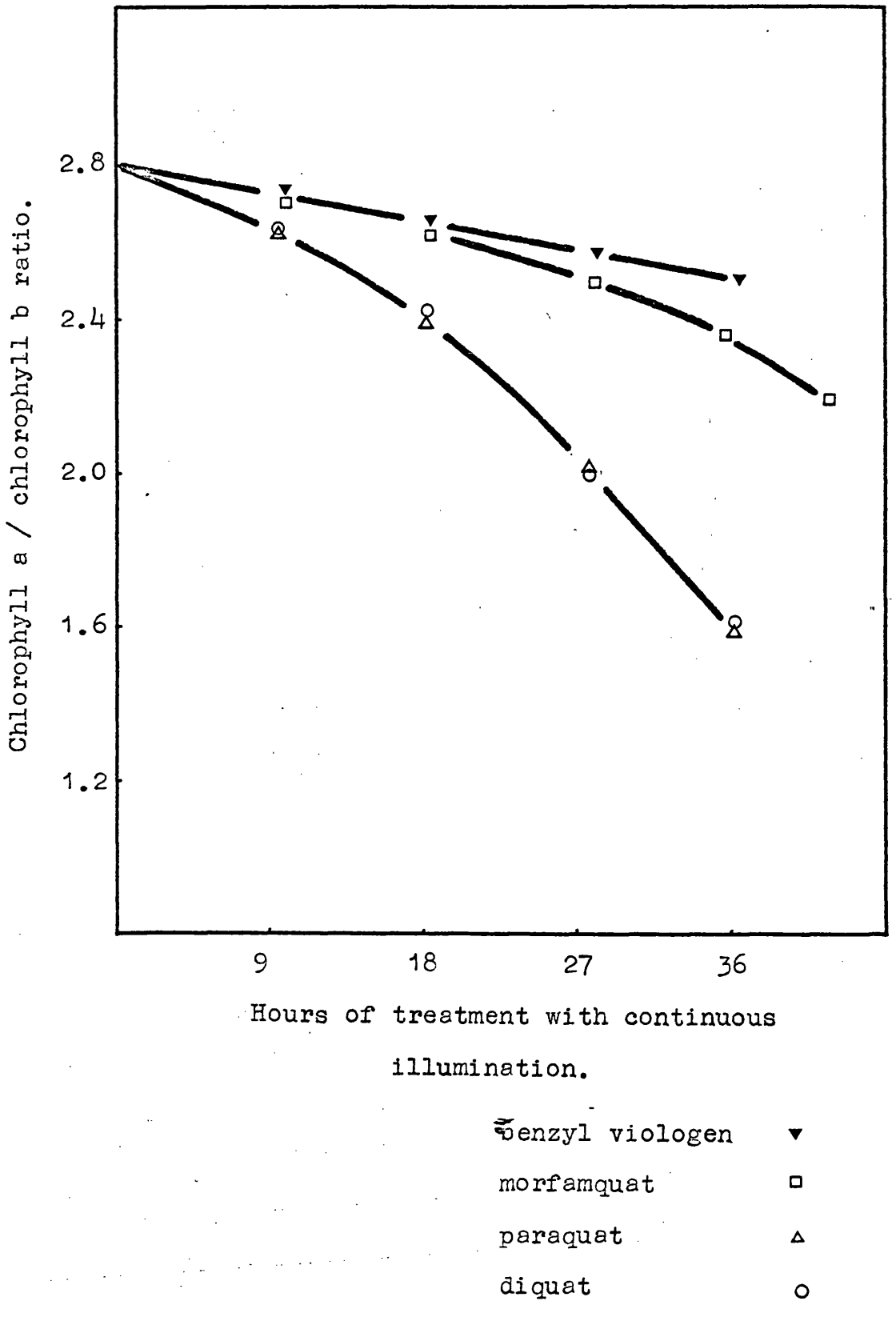


Figure 11. The effect of light and dark periods on the chlorophyll levels of paraquat treated flax cotyledons.

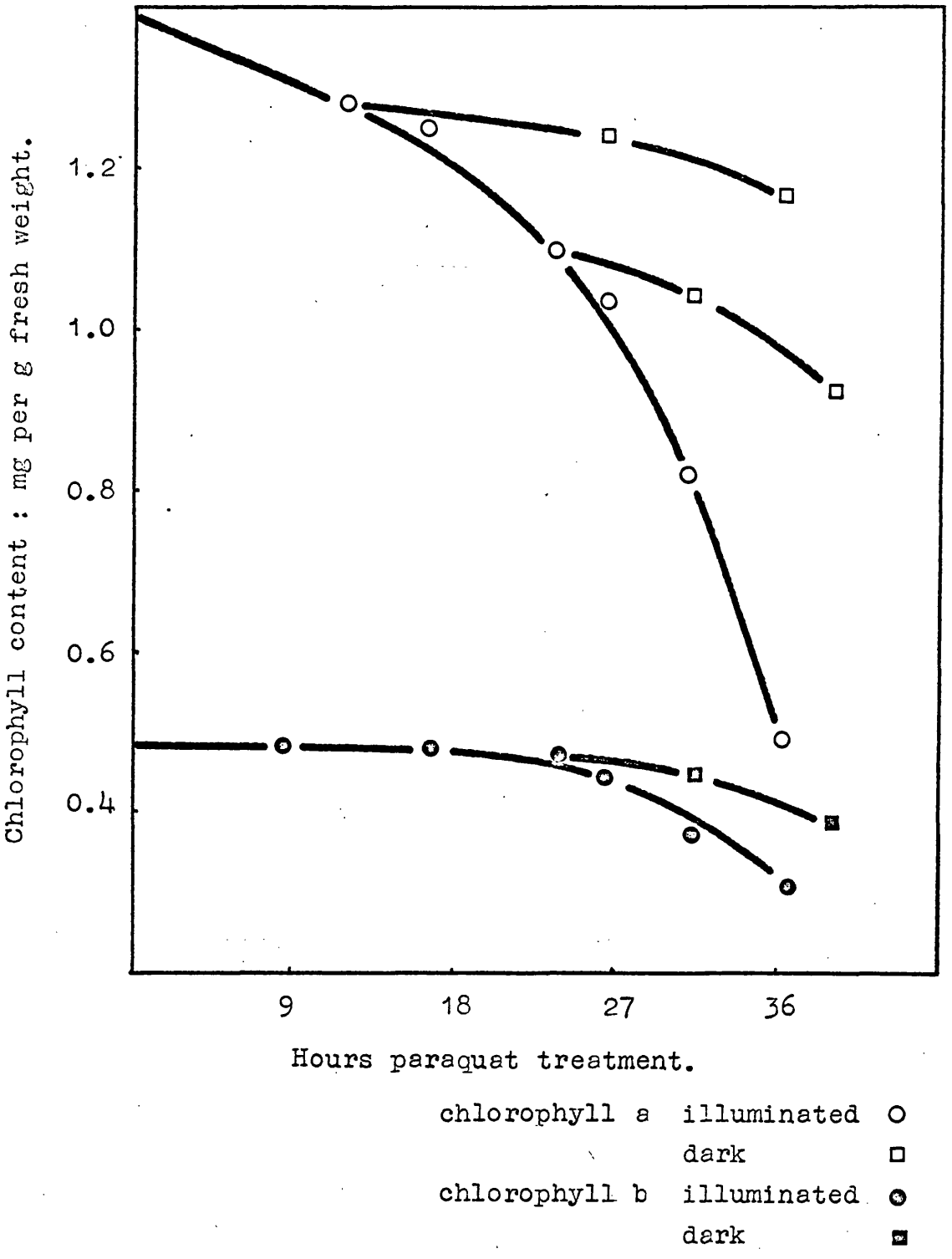
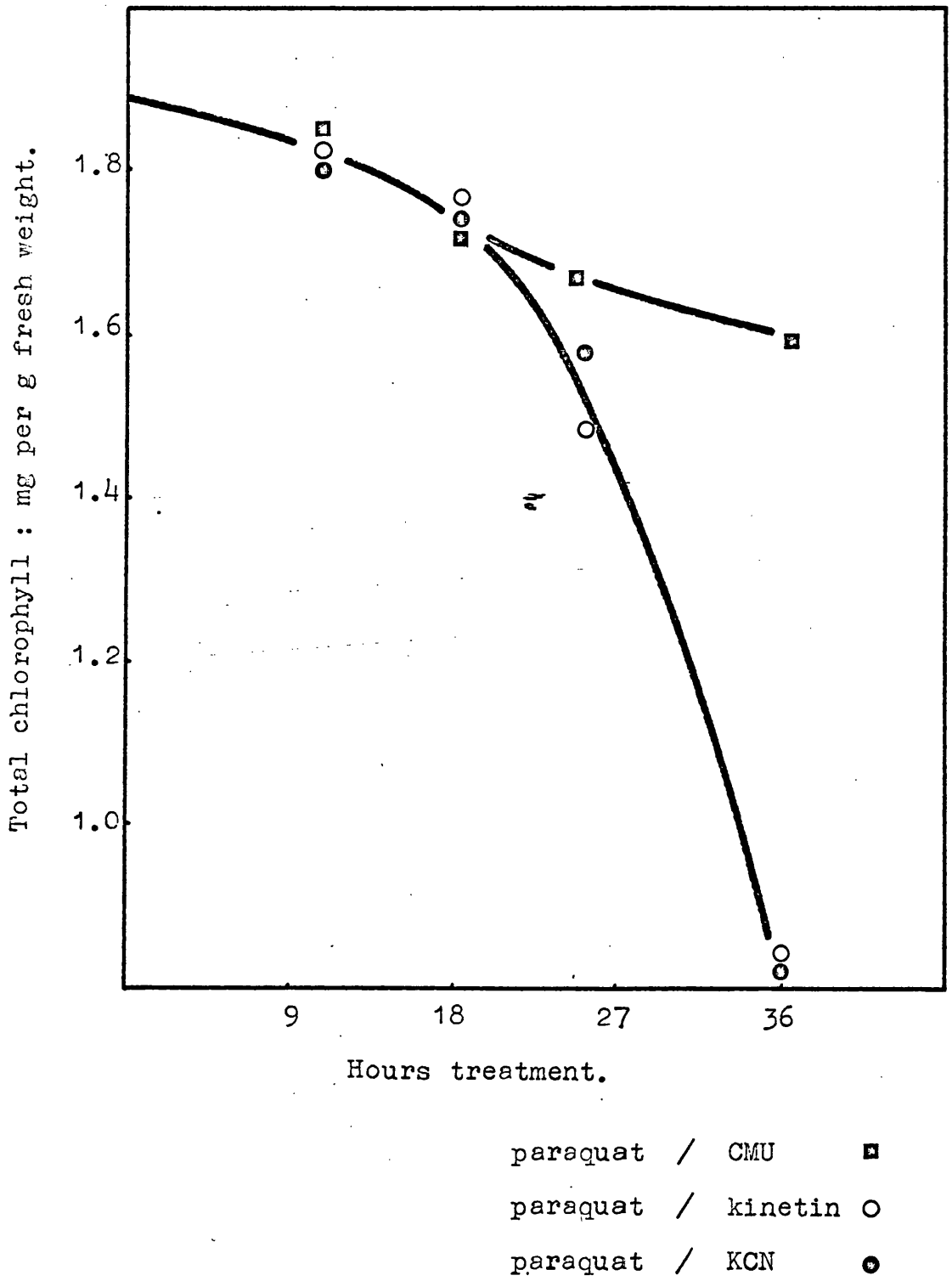


Figure 12. The effect of CMU, kinetin and KCN on chlorophyll levels of paraquat treated flax cotyledons.



Results given below show that the photosynthetic electron transport system was completely inhibited after some 20 hours. As the rapid kill by the bipyridyls is dependent on photosynthesis their direct role must be assumed to be complete after this time. However it is obvious that there was a reduction in the rate of pigment breakdown if the cotyledon leaves were placed in the dark after some 20 hours exposure to paraquat in the light. It could be significant that by this stage there was a drop in the levels of carotenoid pigments, as shown in Figure 13. It has been suggested that the carotenoids may normally play a protective role in preventing chlorophyll photo-oxidation (Koski and Smith, 1951; Sistrom, Griffiths and Stanier, 1956; Rabinowitch, 1956).

The accumulation of pheophytin and pheophorbides, the breakdown products of chlorophyll, was shown by the results presented in Figure 14. This type of breakdown is similar to that observed with other treatments of plant material which affect the pigments, e.g. food processing (Tan and Francis, 1962), pigment chromatography (Strain, 1958).

Figure 13. The effect of paraquat on the level of carotenoids in flax cotyledon leaves.

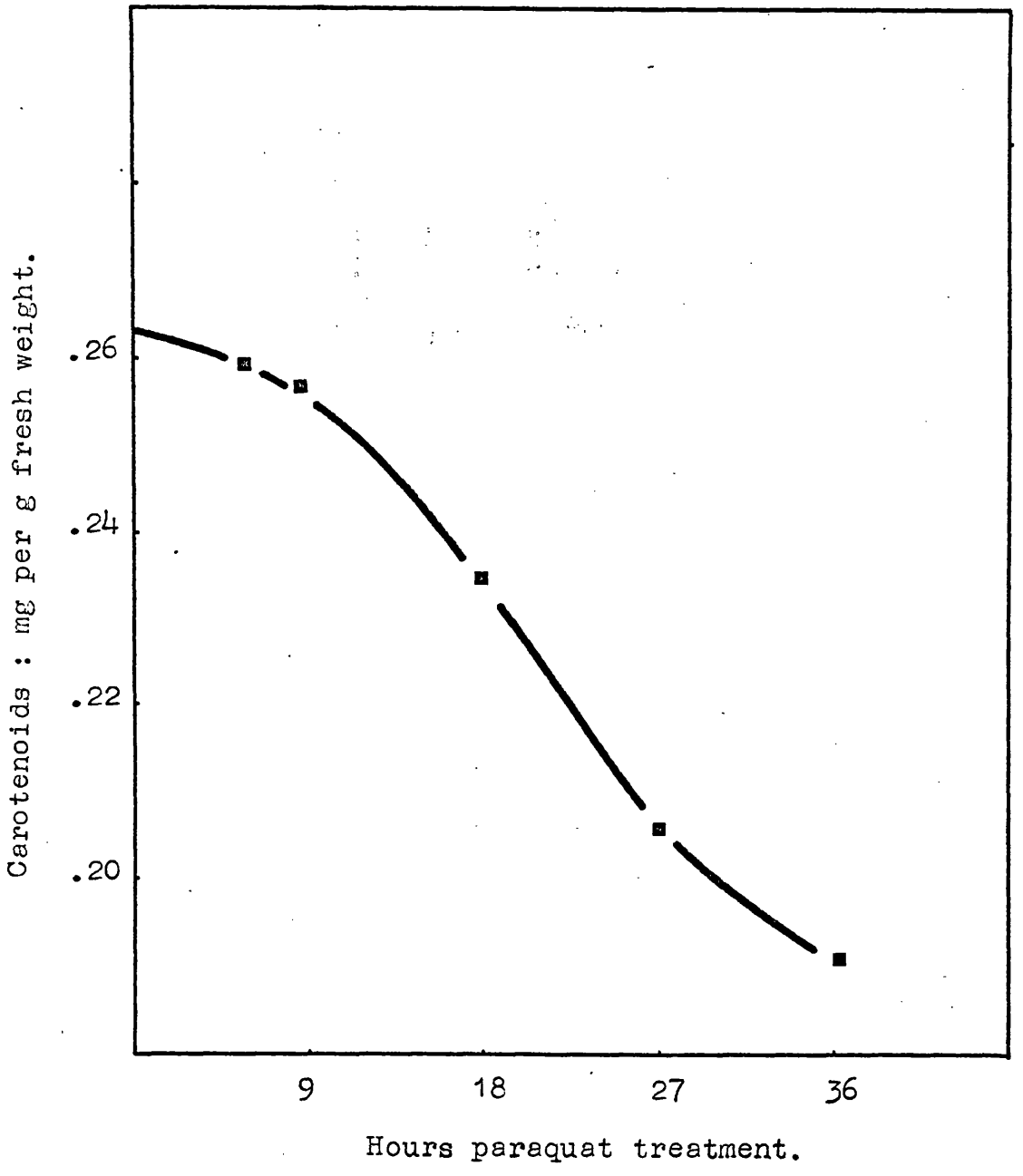
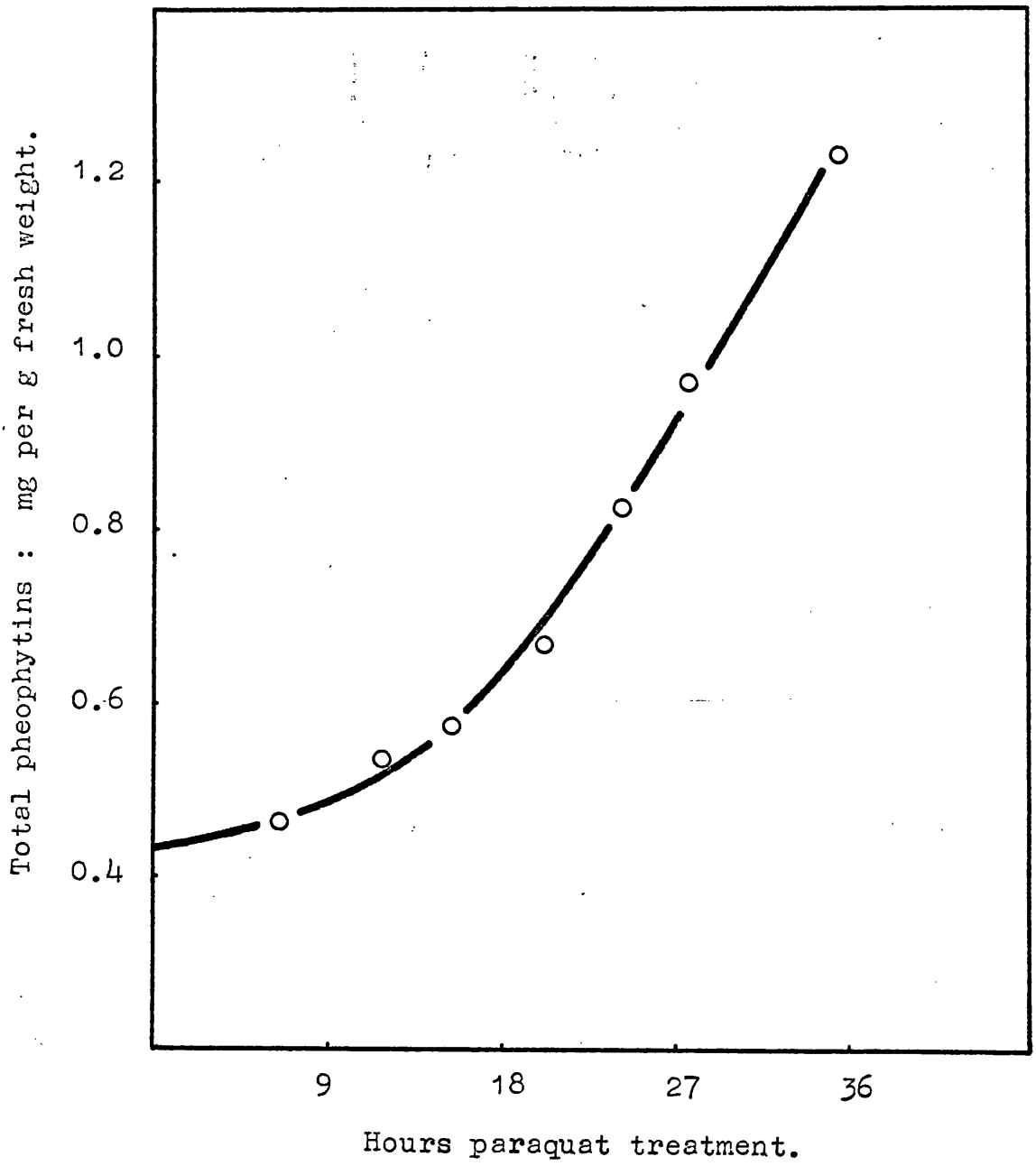


Figure 14. The effect of paraquat on the pheophytin level in flax cotyledon leaves.



2. Lipid analysis

Thin layer chromatography of total lipids extracted from flax cotyledon leaves showed the major components to be mono- and di-galactosyl diglyceride, the phospholipids: phosphotidyl ethanolamine, phosphotidyl choline and phosphotidyl glycerol and neutral lipids, sterols and pigments.

Monoglactosyl and digalactosyl diglyceride were identified for the first time in wheat flour (Carter, McCluer and Slifer, 1956; Carter, Hendry and Stanacer, 1961a & b) and have since been found by several other authors (Benson, Wintermans and Wiser, 1959; Wintermans, 1960; Zill and Harmon, 1962; Kates, 1960) in photosynthetic plant tissues. The galactosyl diglycerides form the largest lipid fraction of the chloroplast and it was suggested (Rosenburg, 1963; Rosenburg and Pecker, 1964) that the galactolipids may have a possible function with respect to the localisation of porphorin structures of the chlorophyll by forming a stable 'lock and key' fit between the phytol chains of the chlorophyll molecules and the fatty acid acyl chain of the galactolipids.

Figure 15, of the separation of total lipid extracts, shows that considerable changes occurred in the lipid composition of the flax cotyledon leaves during the course of the diquat and paraquat treatments. There was a massive breakdown of galactosyl glycerides, phospholipids and pigments. During the course of the

herbicide treatment, components appeared which included pigment breakdown products and three other clearly identifiable compounds.

A spot running at a slightly lower rf than the normal monogalactosyl diglyceride was identified as containing glycerol, galactose and a fatty acid profile similar to that found in the normal digalactosyl diglyceride. This compound was apparent in total lipid extracts of 36 and 60 hour paraquat treated flax cotyledon leaves and was regarded as being a monogalactosyl derivative of digalactosyl diglyceride.

The other two compounds, which ran close together just below the pigment spots, gave colour reactions with periodate-Schiff's reagent and ran at rfs that indicated that they were mono- and di-glycerides. Elution of these spots and subsequent identification of the fatty acid profiles by gas liquid chromatography showed similar patterns to those of the fatty acids of the galactosides. There was one other peak present which corresponded to that of the fatty acid profile associated with flax cotyledon leaf phosphotidyl ethanolamine (Figure 16).

Thus it is suggested that the digalactosyl diglyceride was broken down to give a monogalactosyl diglyceride and this in turn was split, along with the original monogalactosyl diglyceride, to give mono- and di-glycerides. Also the phospholipids, phosphotidyl ethanolamine and phosphotidyl choline were broken down to give glycerides.

Figure 15. Chromatography of total lipid extracts of control and 40 hour paraquat treated flax cotyledon leaves.

A sprayed with phosphomolybdic acid

B sprayed with periodate-Schiff reagents

C sprayed with molybdophosphoric acid

a pigments

b mono and diglycerides

c monogalactosyl diglyceride

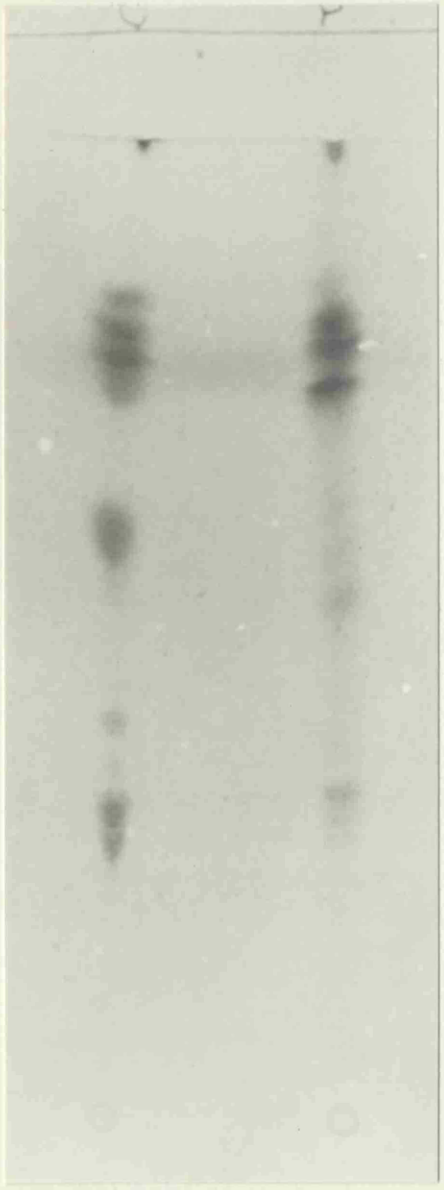
c' " " from digalactosyl diglyceride

d phosphotidyl ethanolamine

e phosphotidyl glycerol

f digalactosyl diglyceride

g phosphotidyl choline



a
b
c
c'
d
e
f
g

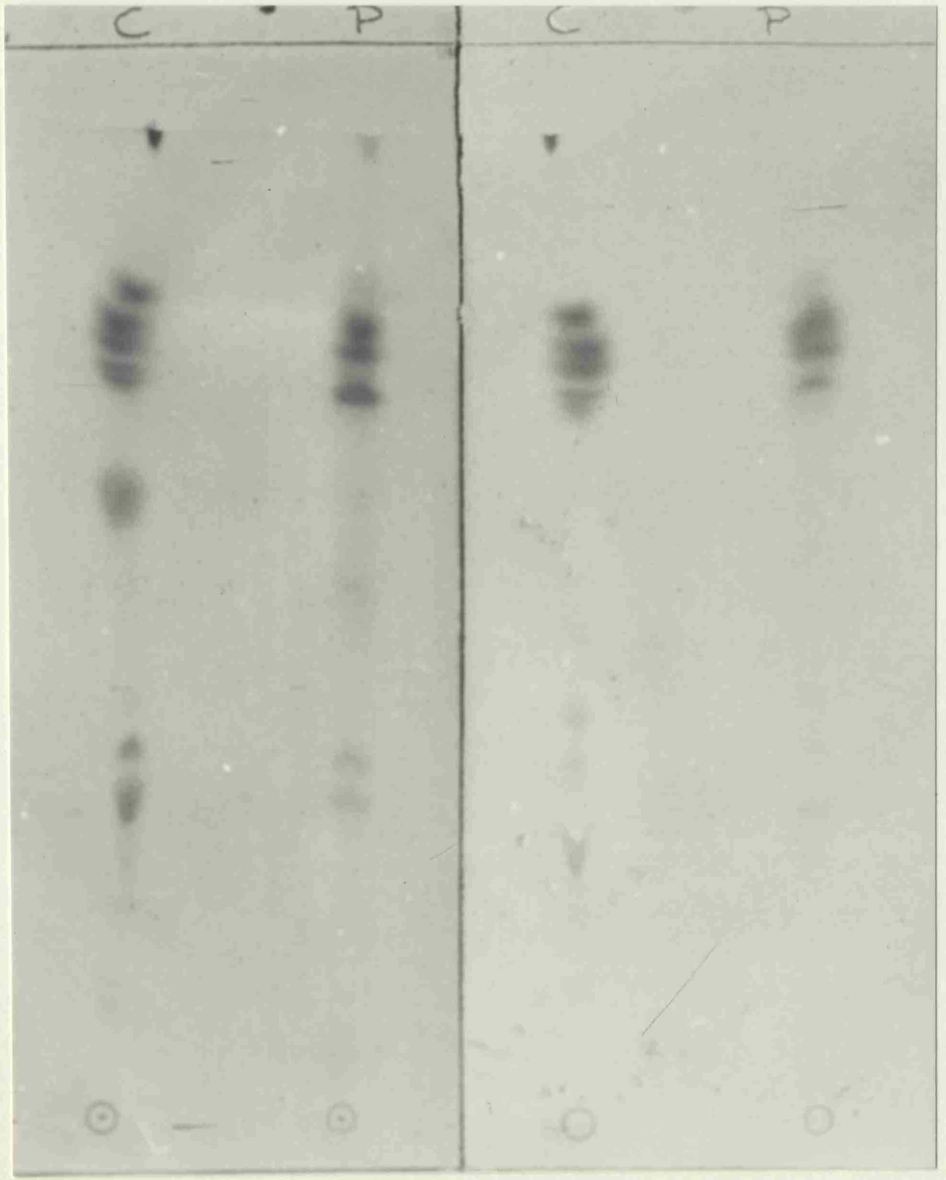


Figure 16. G.L.C. traces of fatty acid methyl esters.

——— from mono and diglycerides of 40 hour paraquat treated flax cotyledon leaves

– – – from monogalactosyl diglyceride

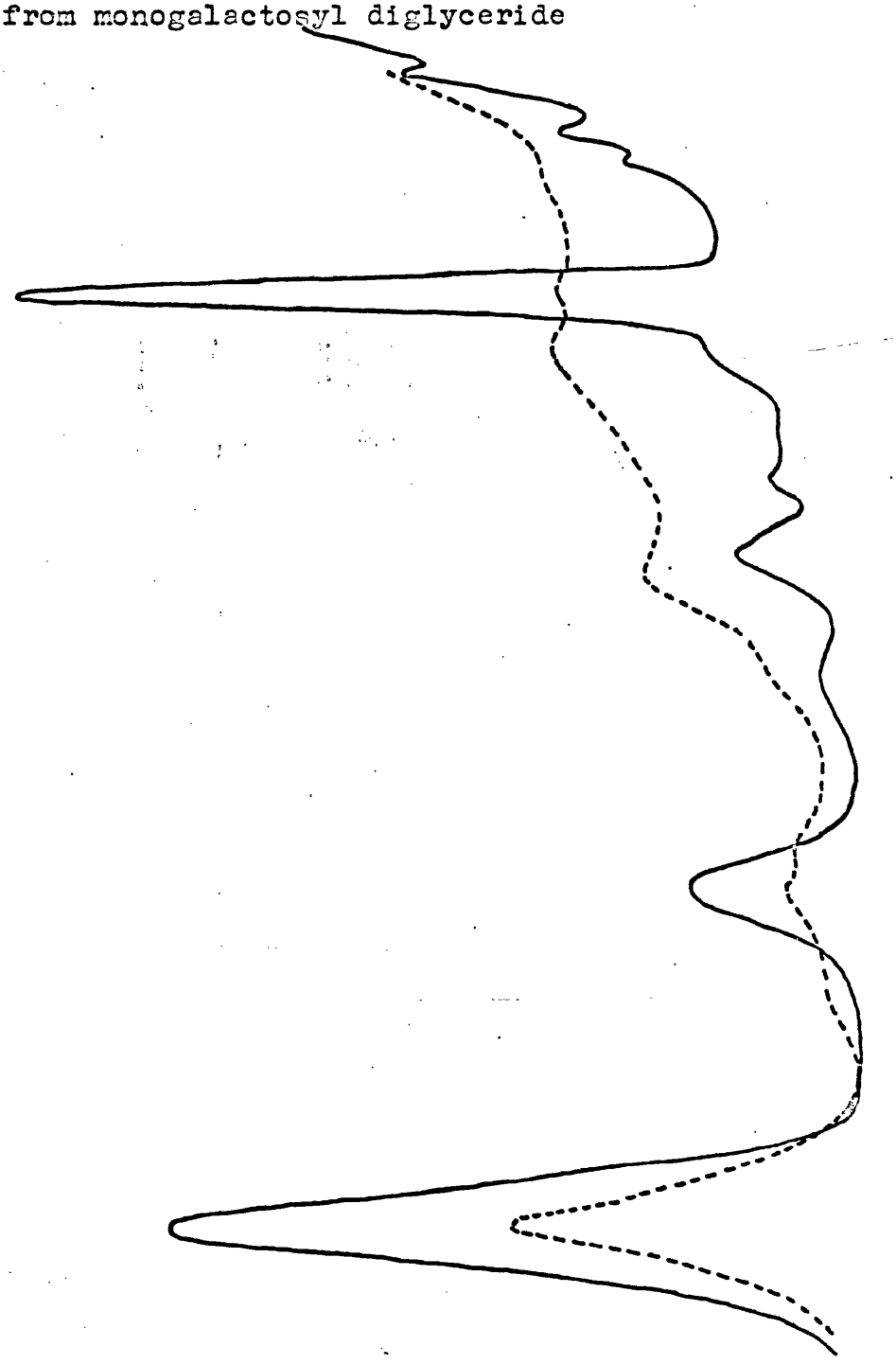
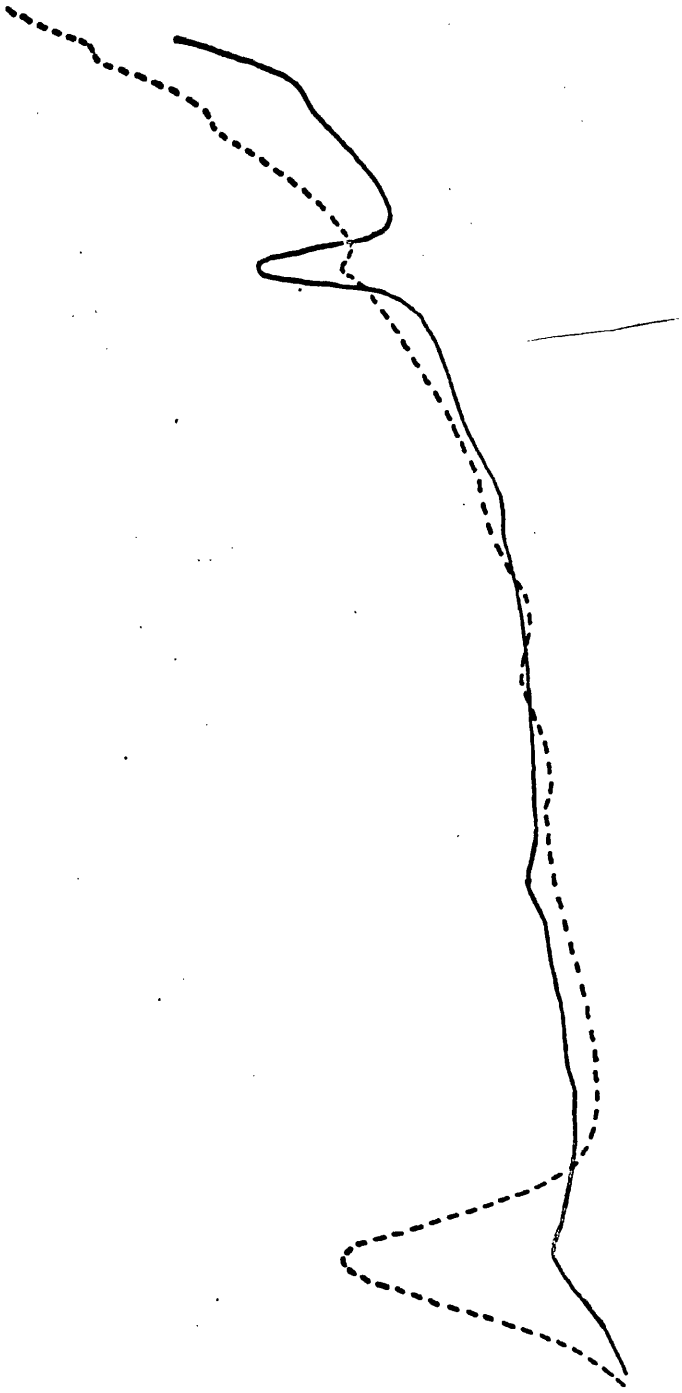


Figure 16.

--- from digalactosyl diglyceride

—— from phosphotidyl ethanolamine



Considerable difficulty was encountered when trying to quantify the changes in the glycolipid components. It was probable that this was due to the loss of some of the water soluble components into the herbicide solution on which the cotyledon leaves were floating. Extraction from the herbicide solution was not considered feasible as the disintegration of the flax was accompanied by a massive increase of the microbial population within the herbicide solution. Because of these difficulties a series of experiments were carried out in which lipids were extracted from cotyledon leaves which had been sprayed with herbicide. Despite this the results of galactose assays of extracts from sprayed cotyldeon leaves gave no clear pattern.

Although it was after approximately 24 hours that changes in the lipid components were shown by thin layer chromatography, some indication of a rapid effect on the lipid of the cotyledon leaves was found in the increase of malondialdehyde. This compound results from the peroxidation of unsaturated lipids (Kohn and Liversedge, 1944; Patton and Kurtz, 1951). In most membrane systems the unsaturated fatty acids are in close association with electron transport systems. Since electron transport systems have been found to produce free radicals (Calvin, 1958), and free radicals initiate the formation of peroxides by hydrogen abstraction, a chain reaction could proceed as shown in Figure 17 (Hardman, 1963; Packer, 1967).

Figure 17. Chain reaction of lipid peroxidation by free radicals.

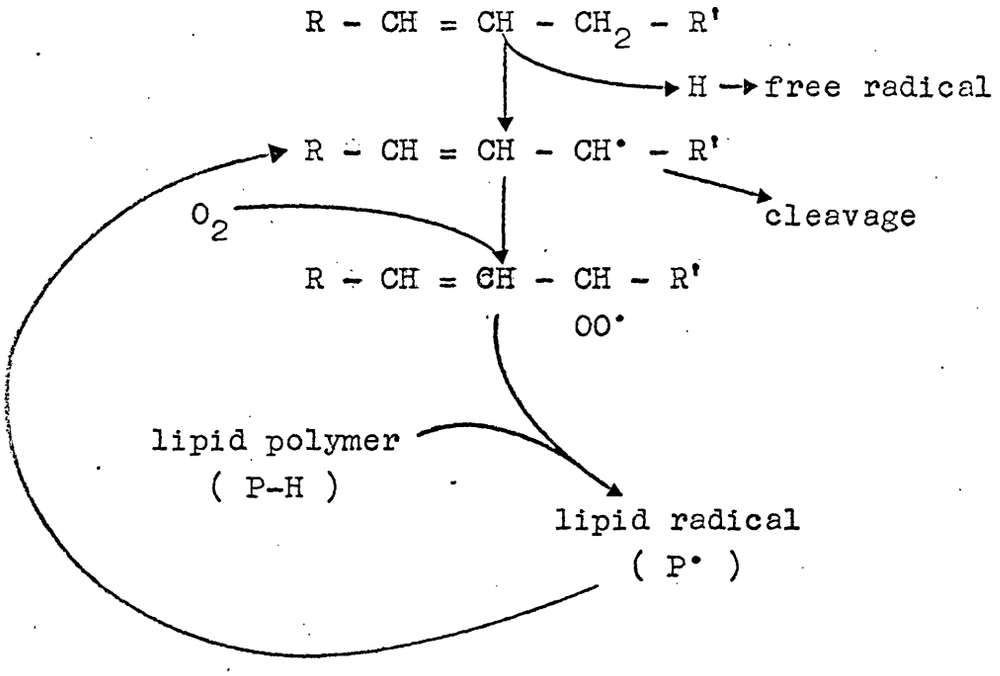
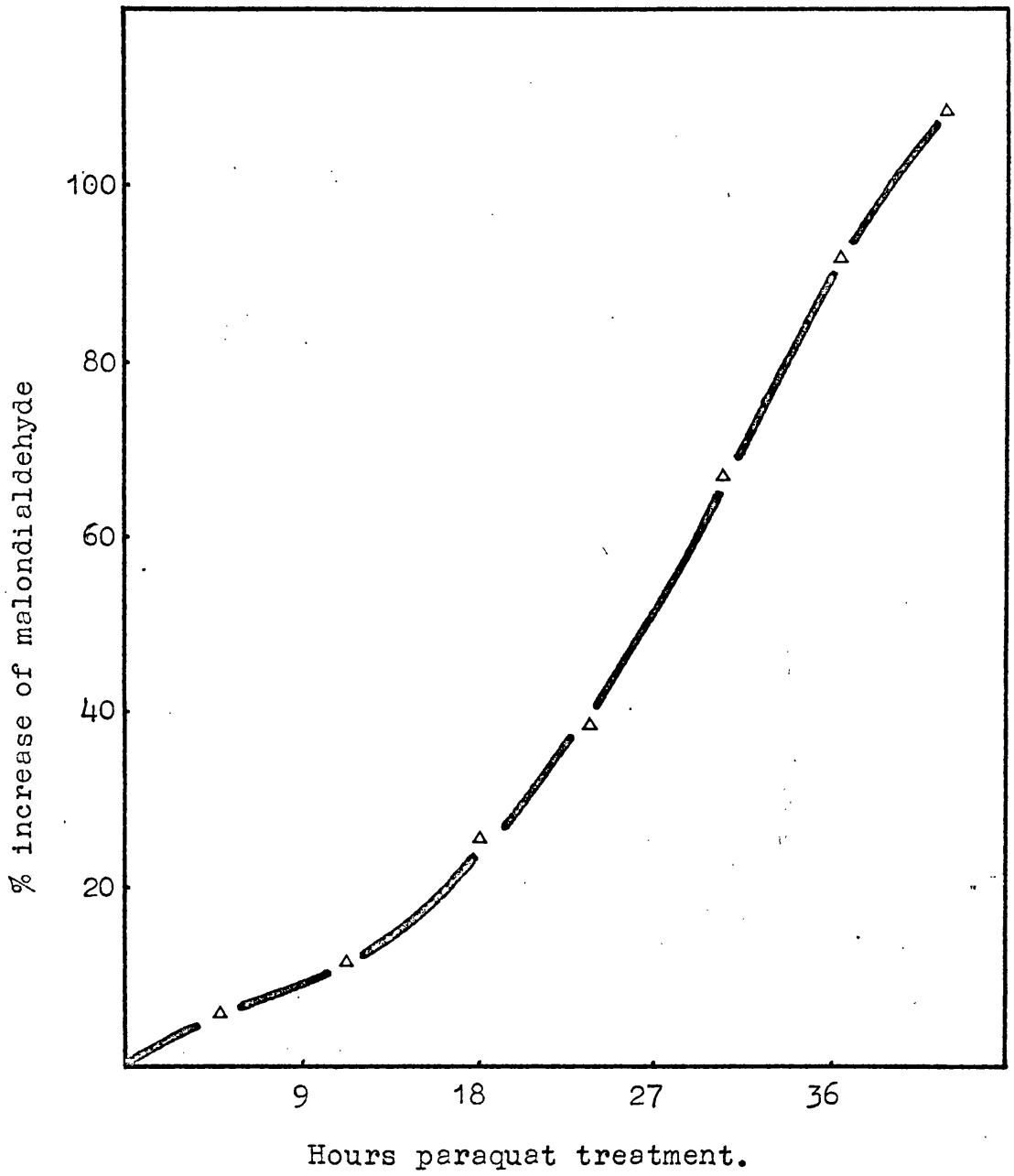


Figure 18 shows that there was a rapid rise in the level of malondialdehyde after only a few hours treatment. The lipid component of the cell is largely involved in the membranes and, as there appeared to be a rapid effect on the lipids during treatment with diquat and paraquat, the permeability of treated cells was examined.

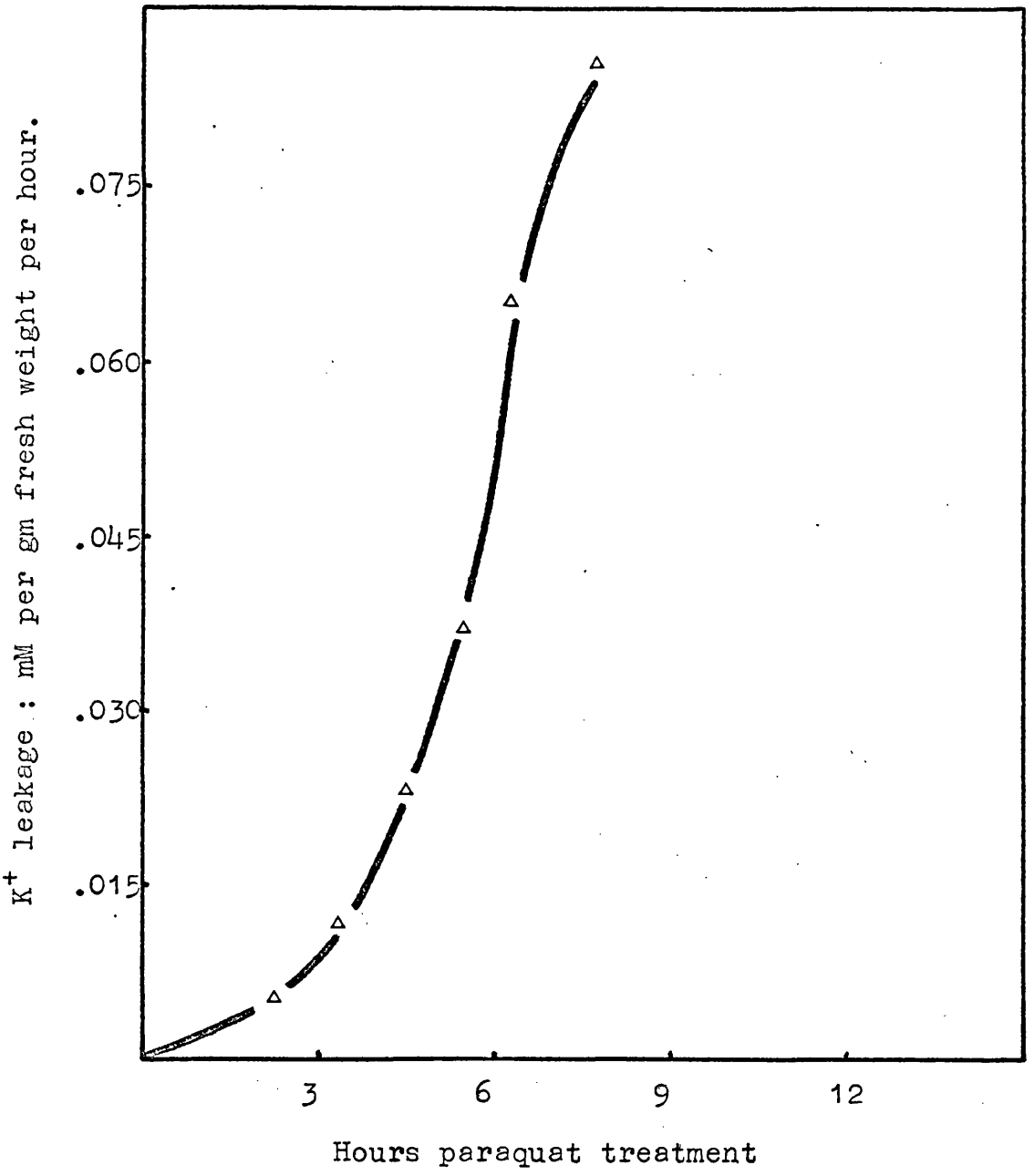
Figure 18. Malondialdehyde in paraquat treated flax cotyledon leaves.



3. Permeability changes

Diamond and Solomon (1959) studied the exchange of intracellular potassium and found that while 98% of the cell potassium was within the vacuole there was a very slow rate of exchange. Eilam (1965) used the leakage of potassium ions from tissue slices as an indicator of membrane deterioration and a similar study was made with diquat and paraquat treated cotyledon leaves. It was found that there was a significant rise in the amount of potassium leakage from the tissue beginning after only 4 hours treatment (Figure 19). Because the potassium is normally contained within the vacuole, it is thought that the efflux that occurred during paraquat and diquat treatment indicated that there was a marked deterioration of the tonoplast.

Figure 19. K^+ leakage from paraquat treated flax cotyledon leaves.



4. Protein changes

Figure 20 shows the change in the level of soluble protein during the treatment of flax cotyledon leaves with paraquat. The drop was similar to that found in senescing flax cotyledon leaves (Figure 21) although the effect was more rapid during the herbicide treatment. Kessler and Monselise (1961) and Hanson and Swanson (1962) linked the degradations during senescence with an increase of degradative actions such as an increase of RNAase, and Lewington, Talbot and Simon (1967) reported the synthesis of some enzymes (eg peroxidase and DNAase) while there was a fall in total protein level. However, during the kill by paraquat and diquat there was no apparent protein synthesis. This was shown by electrophoretic chromatography of the active protein extracted from cotyledon leaves during the course of the kill by the herbicides. With the progression of the herbicide treatment there was a breakdown of the total soluble protein (Figure 20) which was shown by gel electrophoresis as a breakdown of the peaks at approximately equal rates. The changes in the iso-enzyme patterns of catalase were examined in particular, and as shown in Figure 22 there was a differential rate of breakdown but in all cases there was no new catalase production.

Figure 20. The effect of paraquat on total soluble protein of flax cotyledon leaves.

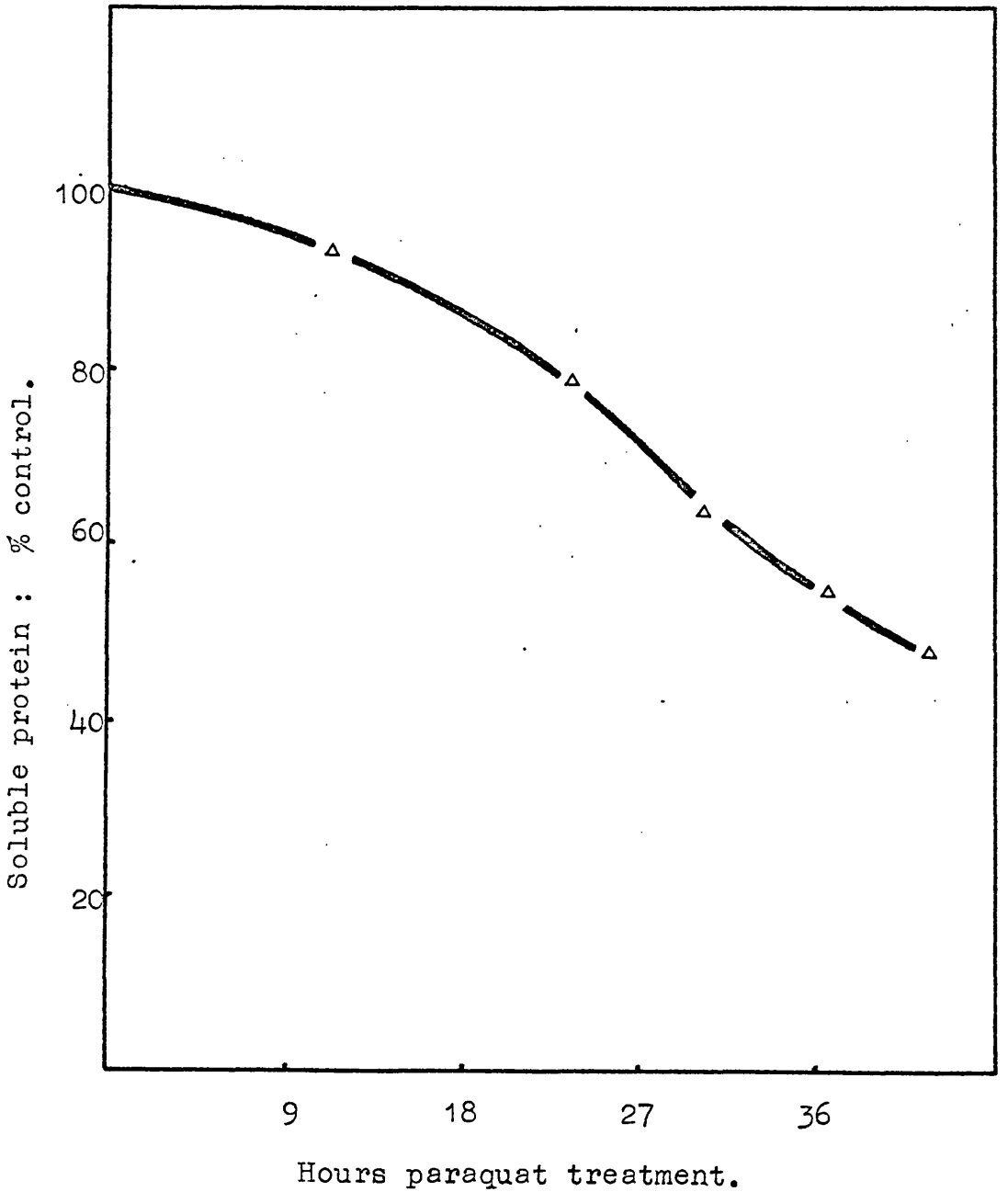


Figure 21. Total soluble protein in senescing flax cotyledon leaves.

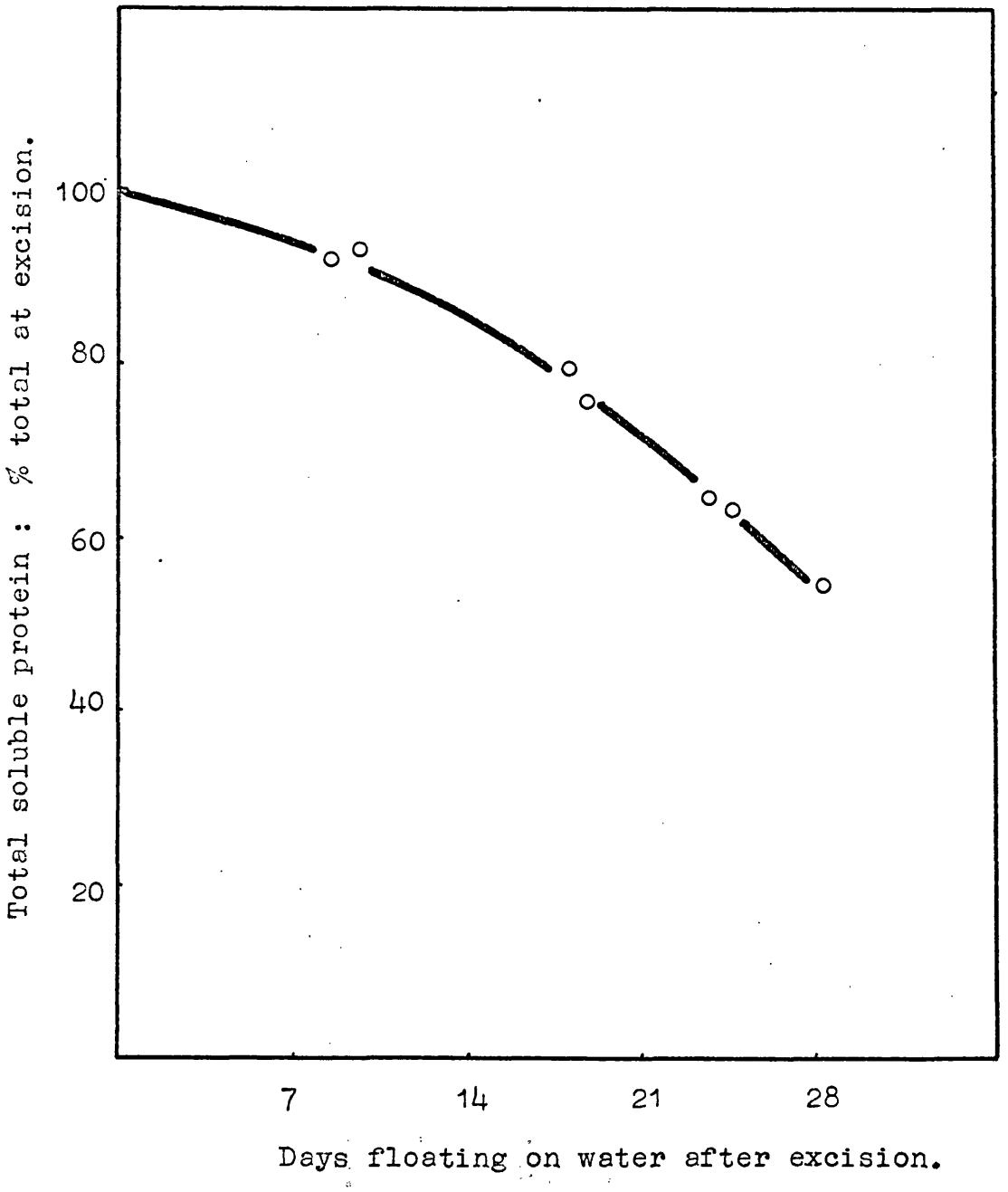
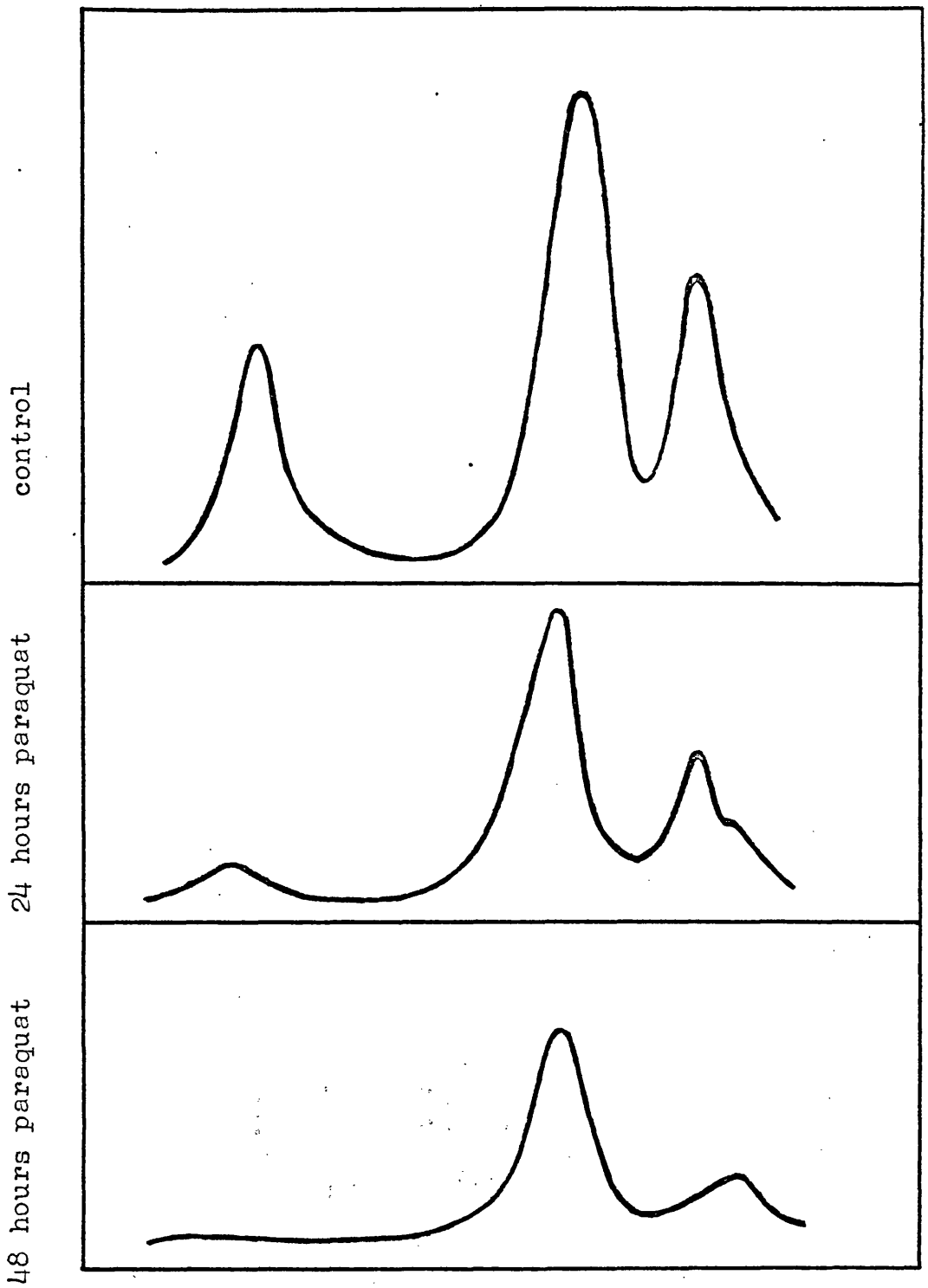


Figure 22. Changes in flax cotyledon leaf catalase iso-enzymes following paraquat treatment.



Densitometer scans of gels after electrophoretic separation of proteins (catalases stained).

Changes in photosynthetic activities

5. Carbon dioxide fixation

Following treatment with paraquat and diquat the flax cotyledon leaves rapidly lost the capacity for photosynthetic carbon dioxide uptake. Figure 23 shows that within $4\frac{1}{2}$ hours from the beginning of the herbicide treatment the flax cotyledon leaves ceased to take up carbon dioxide photosynthetically, and it was found this time was similar in both light and dark. It is probable that it took this length of time for the paraquat to reach all of the chloroplasts and that the inhibitory effect was then immediate. Figure 23 also shows that during this time there was an increase in the rate of respiration as demonstrated by an increase in the carbon dioxide efflux during dark incubation.

6. Photosystem II activity

Although the capacity for photosynthetic carbon dioxide uptake was lost after $4\frac{1}{2}$ hours treatment with paraquat or diquat, chloroplasts isolated from herbicide treated flax cotyledon leaves retained a capacity for light driven electron flow from water for a further period of approximately 18 hours. The exact time to cessation of Hill activity with ferricyanide as the acceptor depended on the temperature and light intensity at which the cotyledon leaves were treated (Figure 24).

Figure 23. Carbon dioxide uptake and evolution by paraquat treated flax cotyledon leaves.

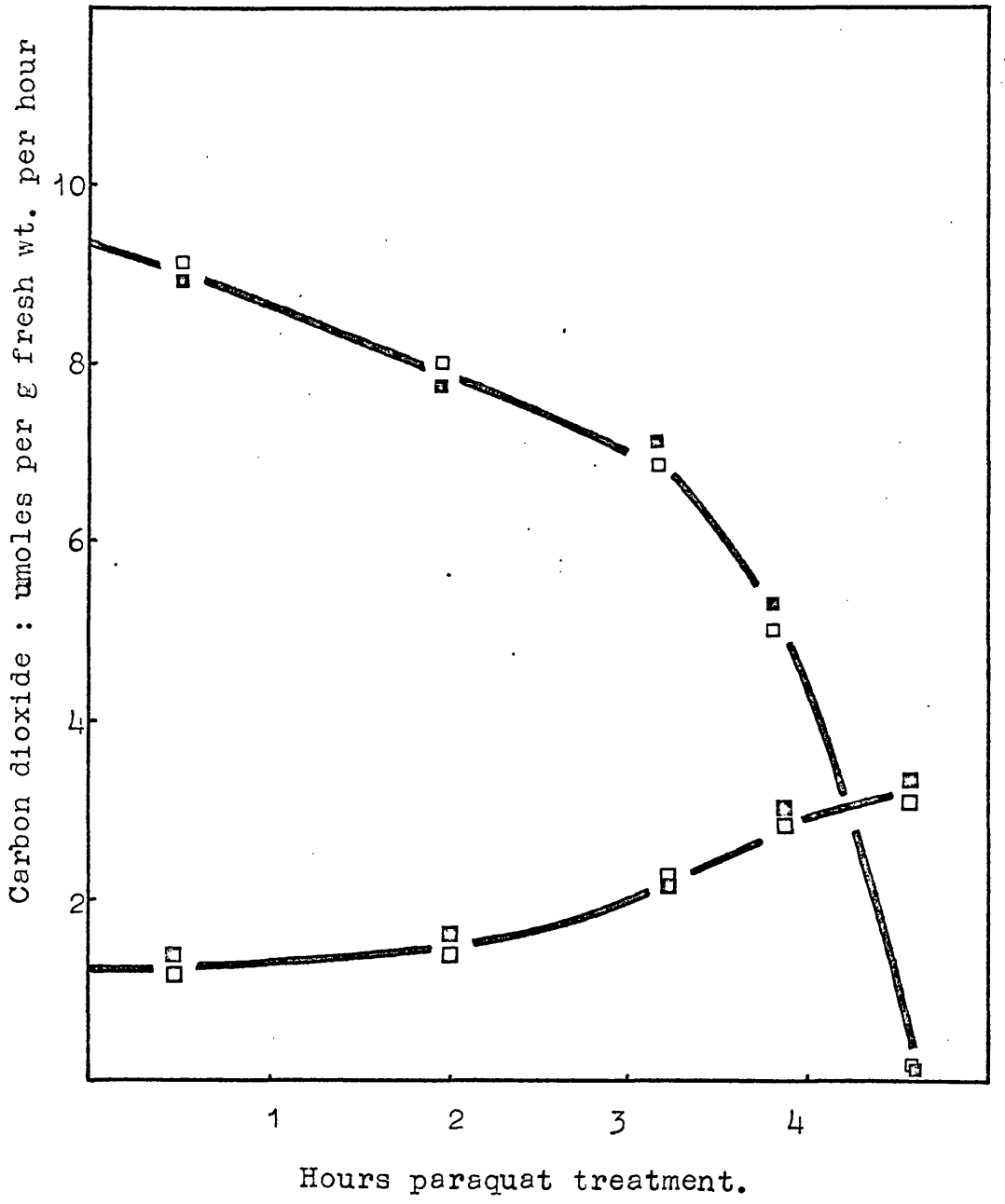


Figure 24. The effect of temperature on the loss of Photosystem II activity during paraquat treatment.

temperature ($\pm$ 1°C)	time to loss of all activity.
11°C	30 hours
16°C	28 hours
20°C	24 hours
24°C	19 hours

Figure 25 shows the course of the decline of ferricyanide reduction by chloroplasts isolated from paraquat treated cotyledon leaves maintained at $25^{o}C \pm 1^{o}C$. The figures are expressed as percentage of the control rate as chloroplasts isolated from untreated cotyledon leaves showed a marked endogenous rhythm with regard to capacity for ferricyanide reduction (Figure 26).

Figure 25 shows the effect of a post-illumination dark incubation on the loss of ferricyanide reduction activity. Although there was a marked reduction in the rate of loss of chloroplast activity when the flax cotyledon leaves were placed in the dark, there was at no time any recovery of lost activity. This was in contrast to the situation in senescence and will be discussed below. When the initial illumination period was increased there was a decreasing effect of the dark incubation. With 15 hours illumination there was no difference in the time to complete loss of ferricyanide reduction activity, whether the cotyledon leaves were maintained in the light or dark. If the cotyledon leaves were floated on paraquat or diquat in the dark prior to illumination, the period of illumination before loss of ferricyanide reduction activity was reduced. The maximum reduction was obtained with a minimum of 4 hours pre-illumination treatment, and this was considered to represent an indication of the time for paraquat to penetrate throughout the cotyledon leaves.

Figure 25. Ferricyanide reduction by chloroplasts isolated from paraquat treated flax cotyledon leaves.

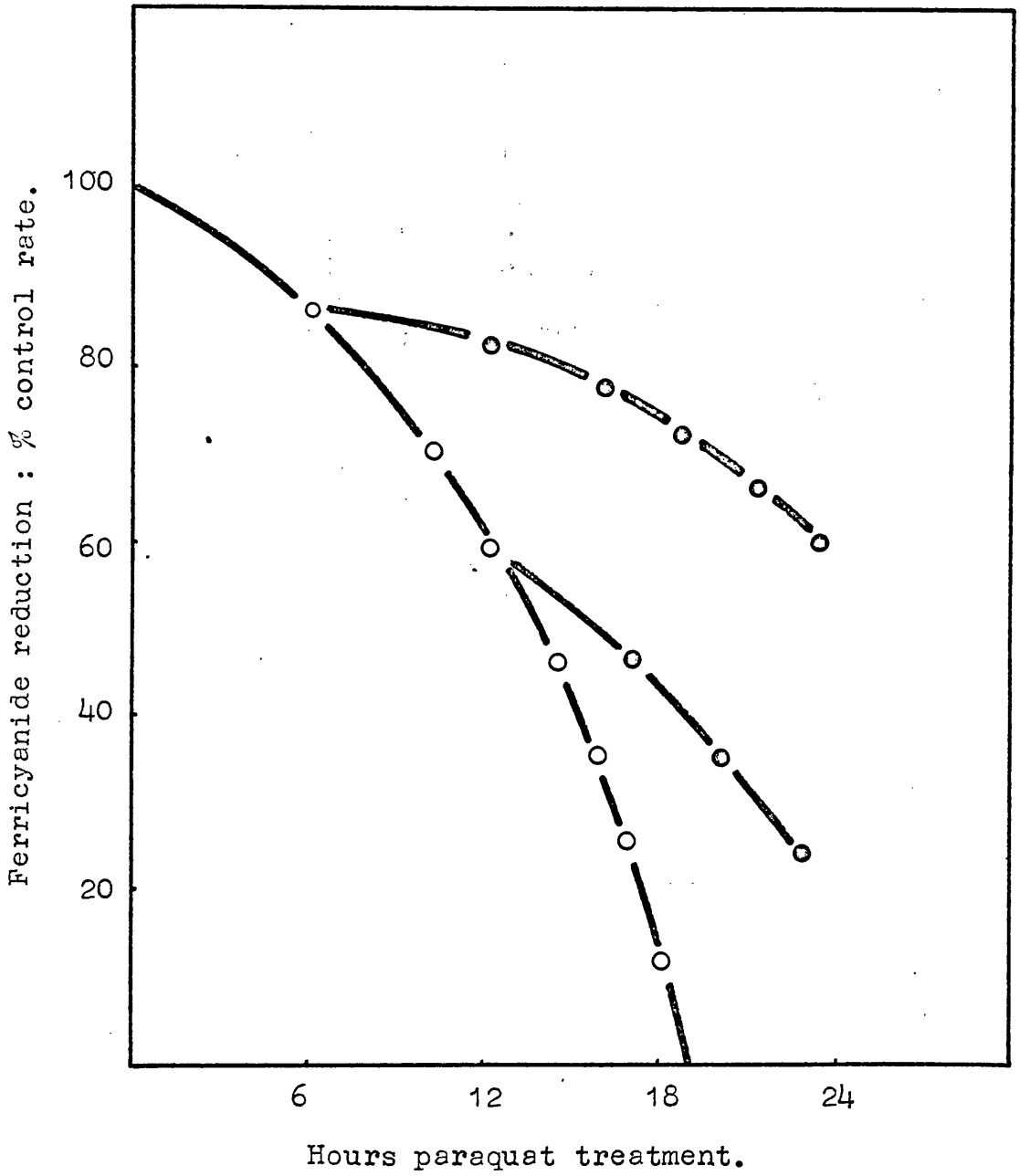
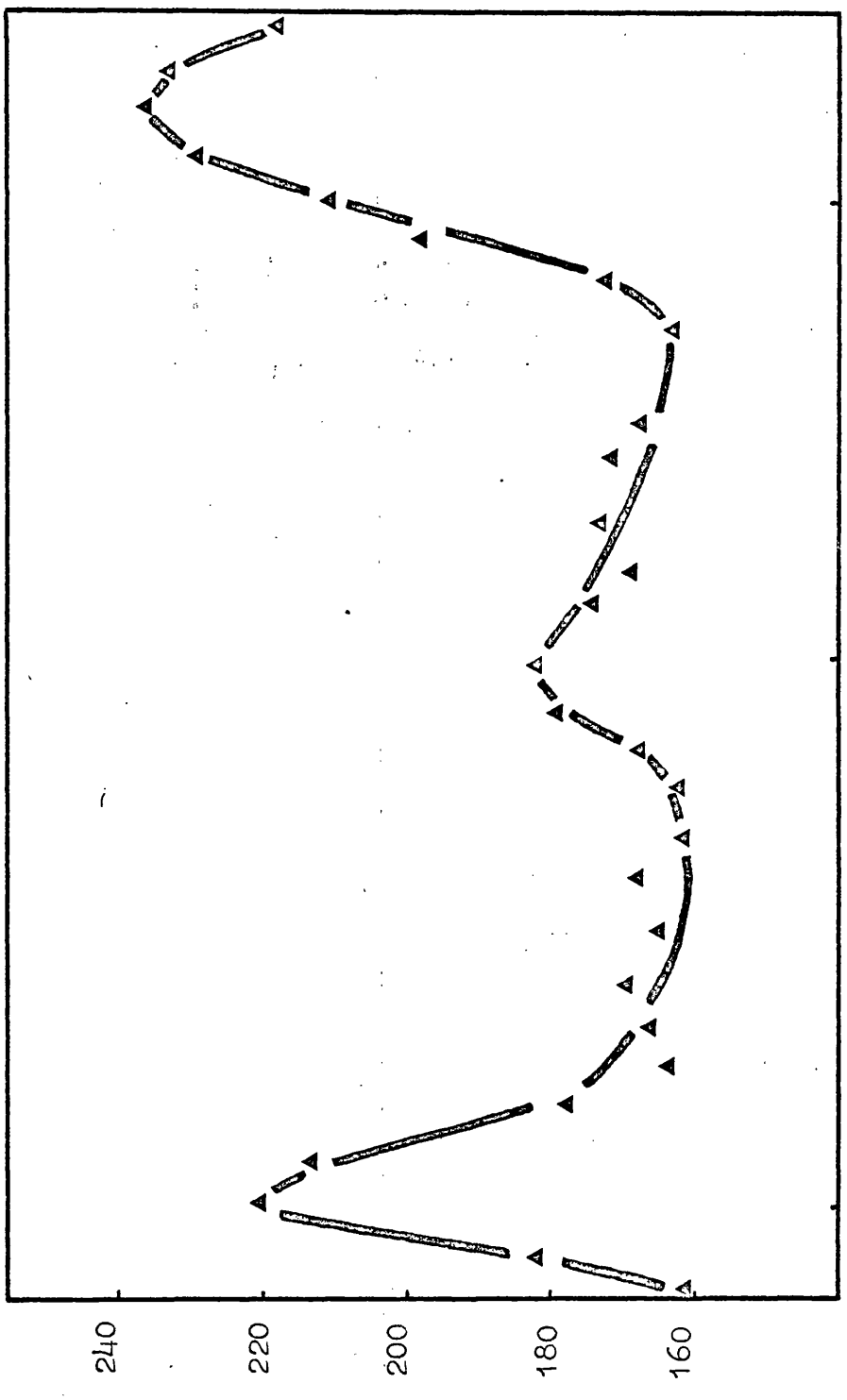


Figure 26. Variation of ferricyanide reduction by chloroplasts isolated from flax cotyledon leaves.



7. Photosystem I activity

Photosystem I activity was measured by inhibiting Photosystem II activity with CMU and measuring the rate of photo-oxidation of ascorbate supplying electrons to Photosystem I via a DCIP couple. Although the chloroplasts isolated from paraquat treated flax cotyledon leaves lost Photosystem II activity within 24 hours, they were able to support a photo-oxidation of ascorbate involving Photosystem I for a longer period (Figure 27). A further significant point was the loss of the requirement for the DCIP couple during the treatment of the flax with paraquat (Figure 28).

It is shown that although photosynthetic carbon dioxide uptake was inhibited within a few hours treatment with paraquat or diquat, photosynthetic electron transport from water continued for up to 24 hours and even after this time the isolated chloroplasts retained a capacity for photo-oxidation of an artificial electron donor via Photosystem I.

Figure 27. Ascorbate photo-oxidation by chloroplasts isolated from paraquat treated flax cotyledon leaves.

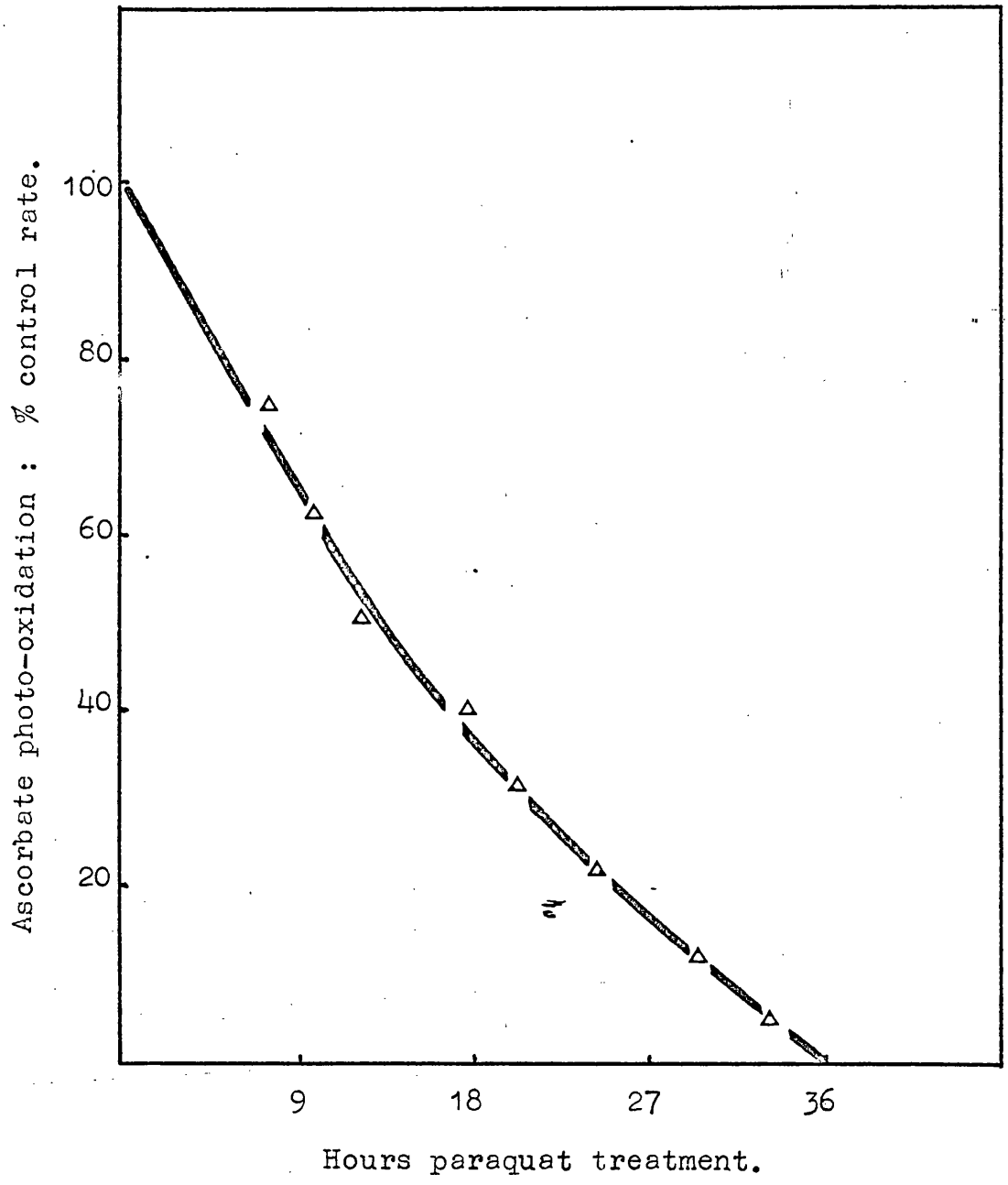
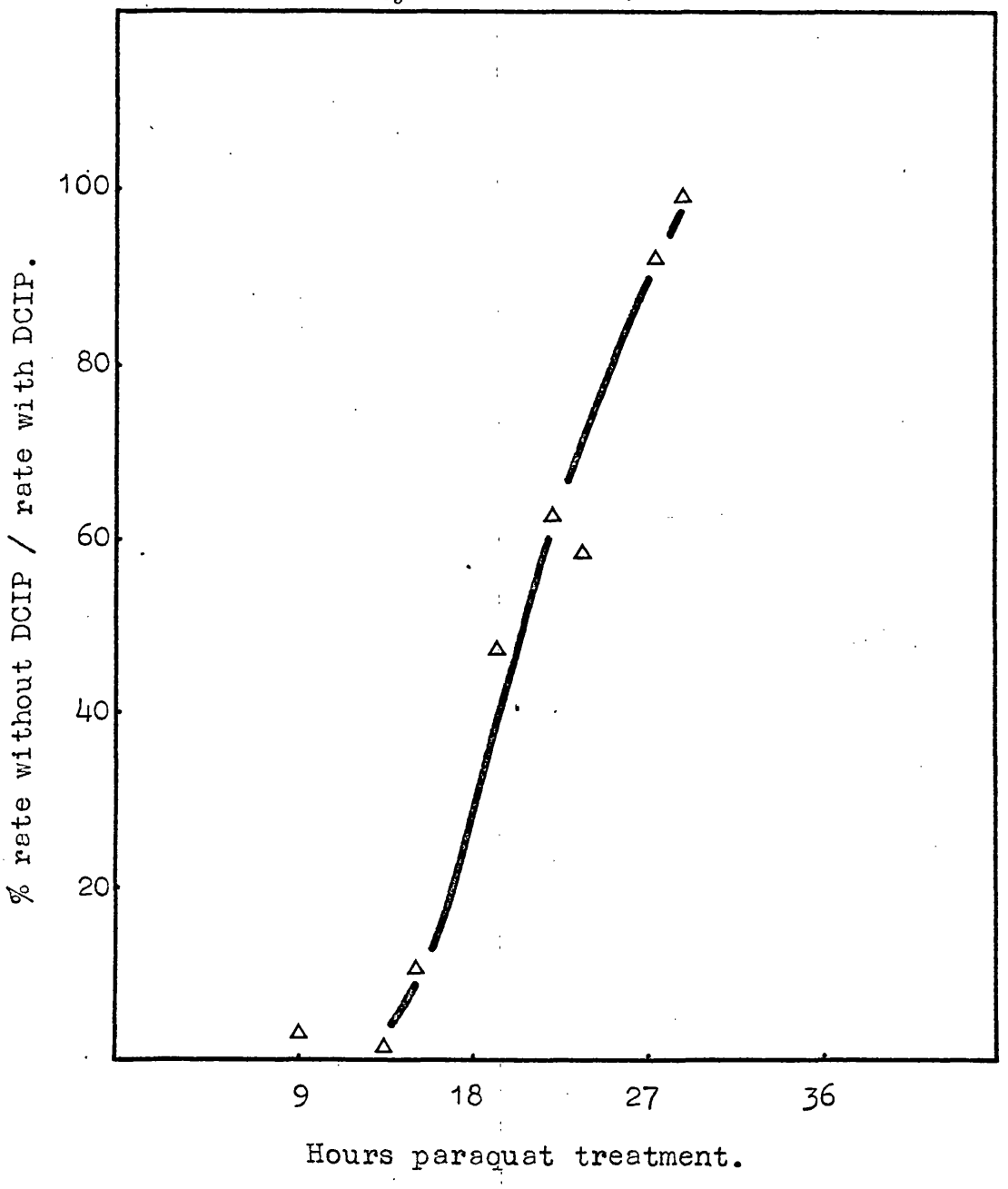


Figure 28. Requirement for DCIP in ascorbate photo-oxidation by chloroplasts from paraquat treated flax cotyledon leaves.



Structural changes

8. Light microscope studies

Light microscopy showed that during the treatment of flax cotyledon leaves with paraquat and diquat there were considerable changes in the internal structure of the mesophyll cells. The normal appearance of the cell with the chloroplasts lying close to the cell wall (Plate 2A) was lost and there appeared to be an aggregation of cell components in the middle of the cell (Plate 2B). There was no change in appearance under the light microscope of cells from cotyledon leaves which had been floated on paraquat in the dark for 60 hours (Fig. 2C).

9. Electron microscope studies

The electron micrographs taken of sections of paraquat and diquat treated flax cotyledon leaves show similar changes to those seen under the light microscope, although with far more detail as to changes in organelle structure. By floating the cotyledon leaves on solutions of paraquat and diquat, there was a lower rate of desiccation of the material and this proved useful as desiccated material was found to be difficult to embed successfully (Kent, personal communication).

It was important that the cells examined for each treatment were from comparable positions in the cotyledon leaves. The area of cells taken for examination was the upper edge of the spongy mesophyll. It was considered that these cells would not be affected by any 'drowning'

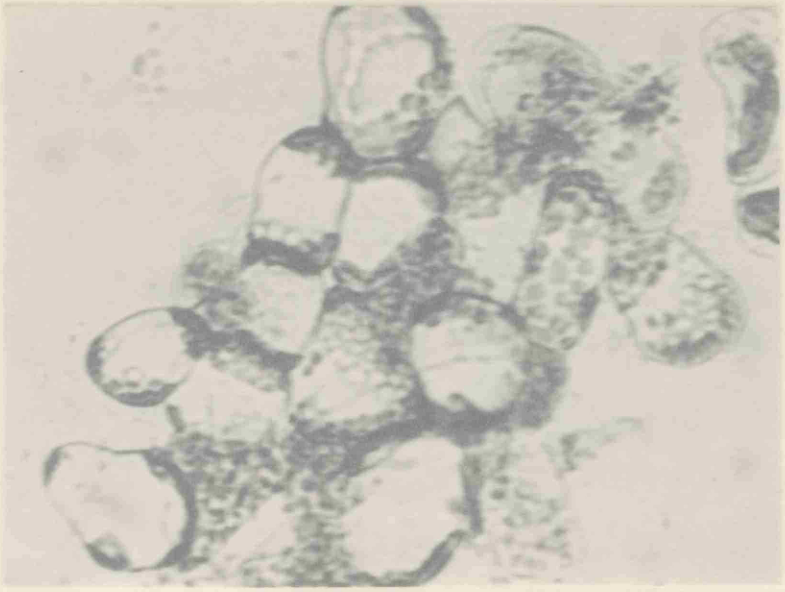


Plate 2A

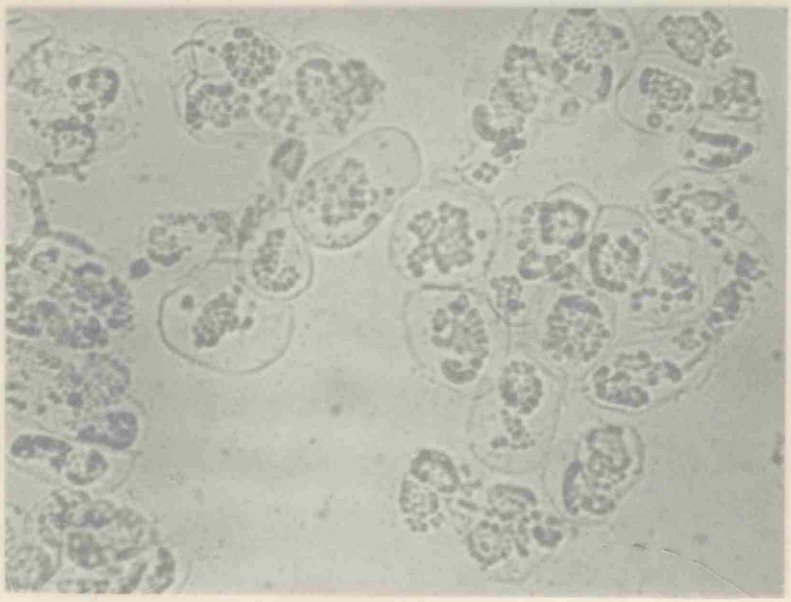
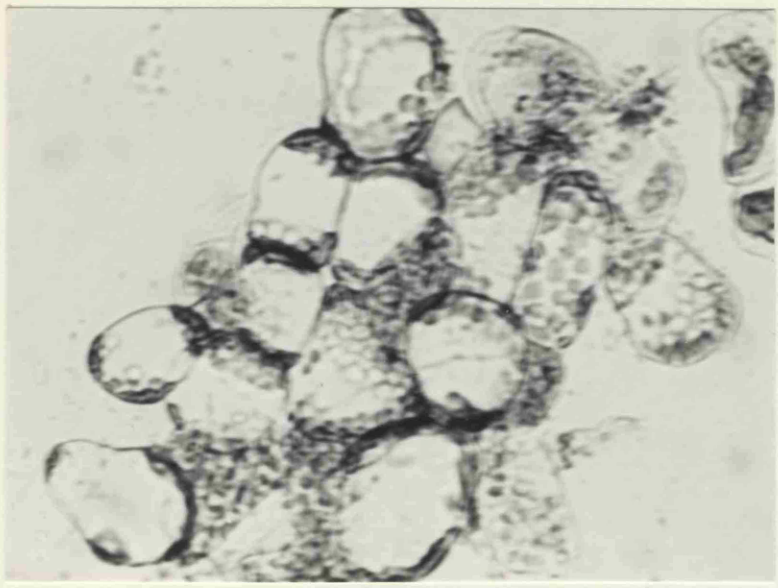
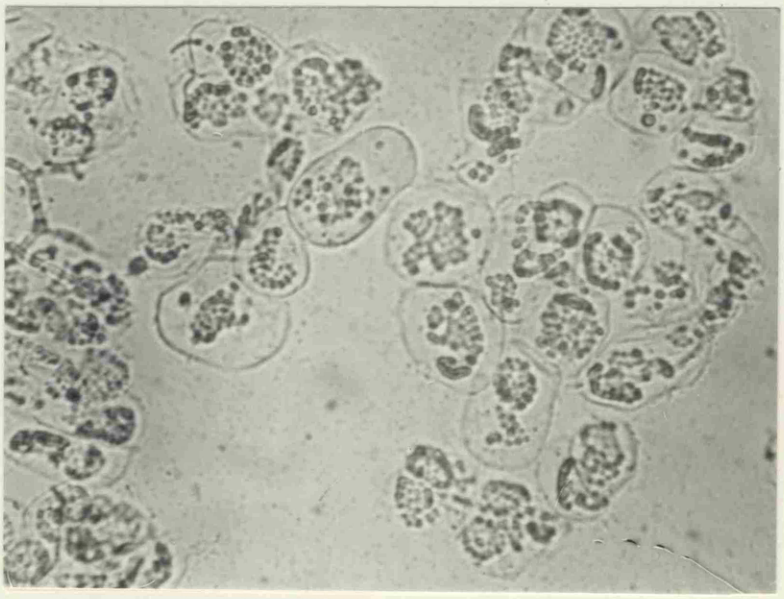
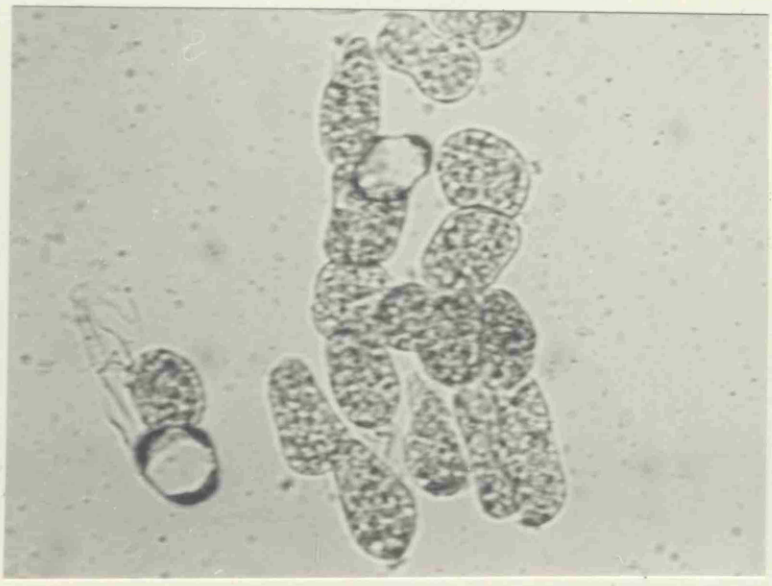


Plate 2B

Plate 2C







which could have occurred with the cells actually in contact with the herbicide solutions. This standardisation of the cells examined was imposed because of possible differing times to saturation with the herbicide solutions, differing degrees of water balance and also different photosynthetic rates of the various types of cell. The times to the same stage of breakdown consequently varied with the different parts of the cotyledon leaves.

In Plates 5 to 14 the times to the various stages of breakdown refer only to the upper spongy mesophyll cells.

Plates 3 and 4 show the appearance of the normal cotyledon leaf cells. The chloroplasts, surrounded by a double membrane, are seen to be arranged close to the cell wall. The plasmalemma is adjacent to the cell wall and the cytoplasm is limited by the tonoplast which in many places lies close to the outer membrane of the chloroplasts. The mitochondria show a double membrane and cristae within. The main features of the chloroplasts are the presence of starch grains, the organisation of the thyllakoids, sometimes with a few associated osmiophilic globules, and an area of nucleic acid particles. In a few micrographs crystals of protein can be seen within the stroma.

One of the first changes in the cell following treatment with diquat or paraquat involved the tonoplast. Plates 5, 6 and 7 show a progression of the disintegration of this membrane which seemed to start with the formation of blisters which often first appeared where the tonoplast ran close to the chloroplast outer membrane. This occurred after approximately 6 hours. At the same time there was an effect on the plasmalemma (Plates 4, 5 and 6) which lost its close alignment to

the cell wall. Plates 5 to 14 show the progression of breakdown of cellular organisation and the destruction of the cell organelles. From about 6 hours treatment the cell mitochondria showed swelling and as they were not apparent in micrographs of longer treatments were thought to have ruptured. The tonoplast was not apparent in any of the micrographs of more than 6 hours treatment and is thought to have disintegrated. Another conspicuous feature during the course of the herbicide treatment was the breakdown of the grana organisation within the chloroplasts and an increase in the number and size of osmiophilic globules, a feature also noticeable during the onset of senescence (Ikeda and Ueda, 1964; Toyama and Ueda, 1965). There was also a change in appearance of the nucleic acid particles within the chloroplast stroma.

10. Production of protoplasts from paraquat treated flax cotyledon leaves, and the action of paraquat on greenleaf protoplasts.

It was found that protoplasts could be prepared from cotyledon leaves which had had less than 4 hours paraquat treatment in the light, but after this period it was not possible.

When protoplasts from untreated flax cotyledon leaves or pea leaves were maintained in a buffered osmoticum with or without paraquat, there was a great loss of protoplasts with paraquat when illuminated. Only preliminary experiments were carried out but these indicated that after about 4 hours green leaf protoplasts were disrupted.

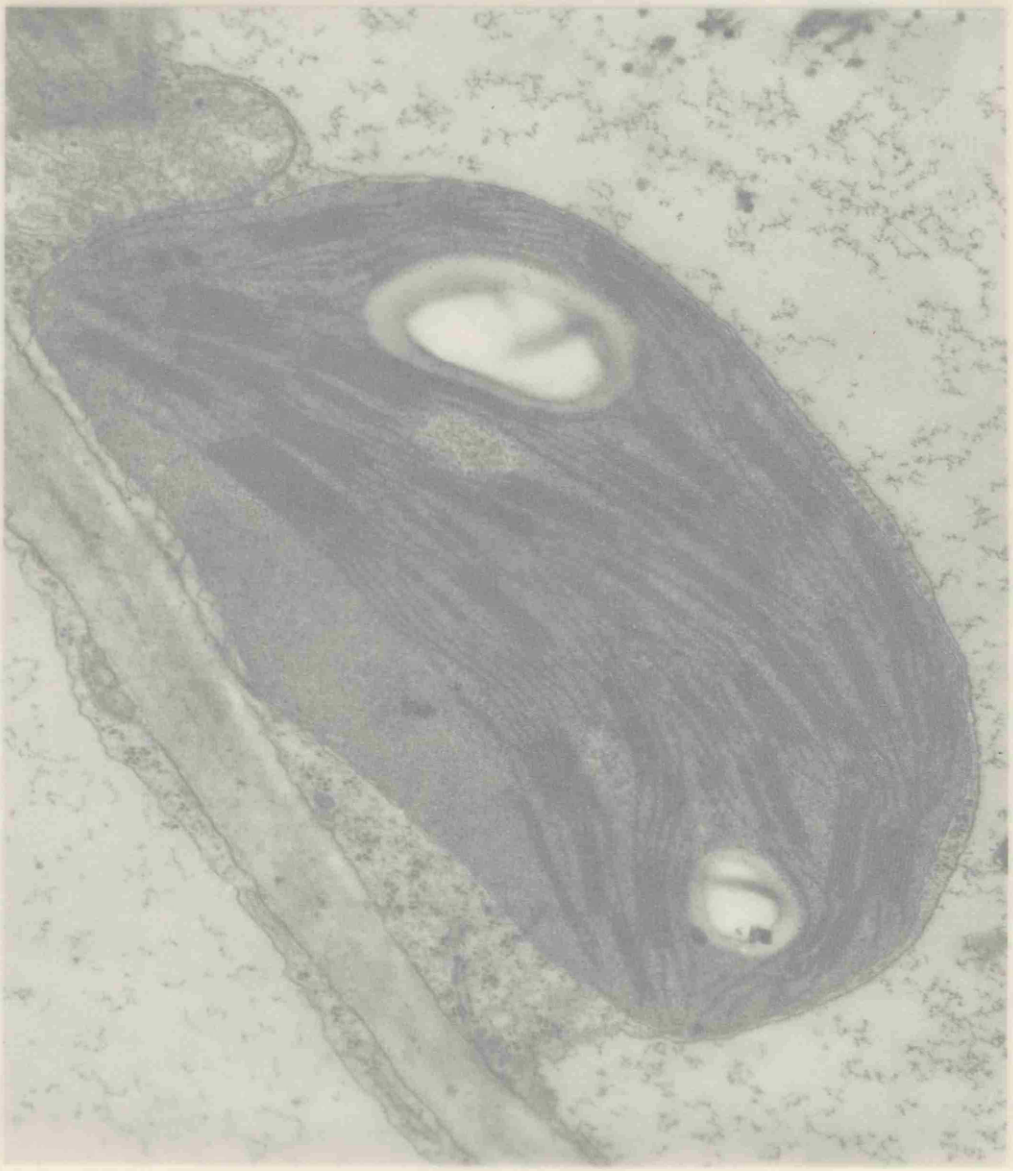
Plate 3. Control flax cotyledon leaf cells.

10 μ.

Note: chloroplasts, with starch granules, and nucleus lie close to the cell wall; mitochondria present in the cytoplasm.

10 μ.

Plate 4. Chloroplast and mitochondrion in control flax cotyledon leaf cell.

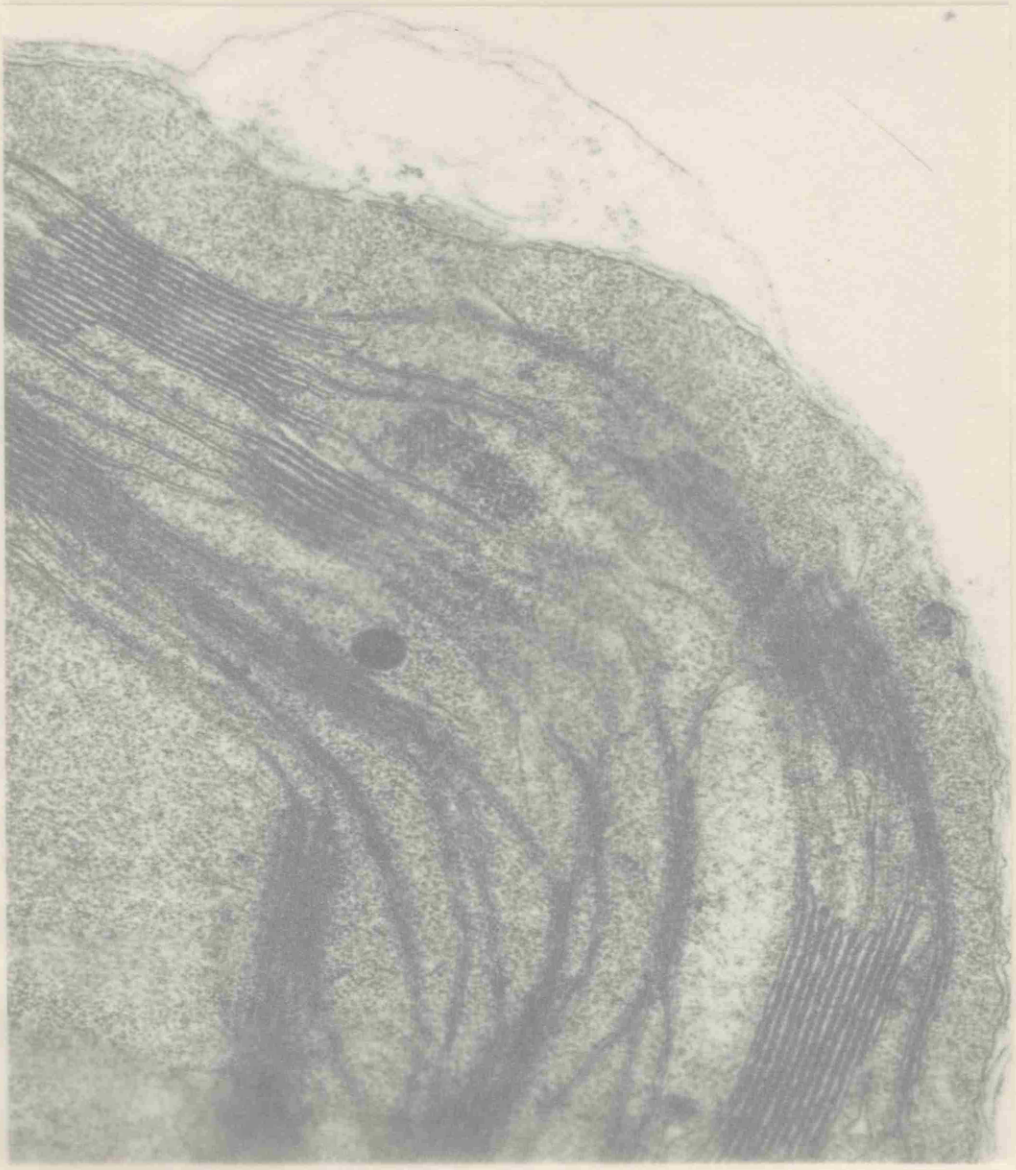


1μ.

Note: intact tonoplast lying close to chloroplast double membrane; ordered grana.

1 μ.

Plate 5. Effect on tonoplast of 6 hours paraquat.

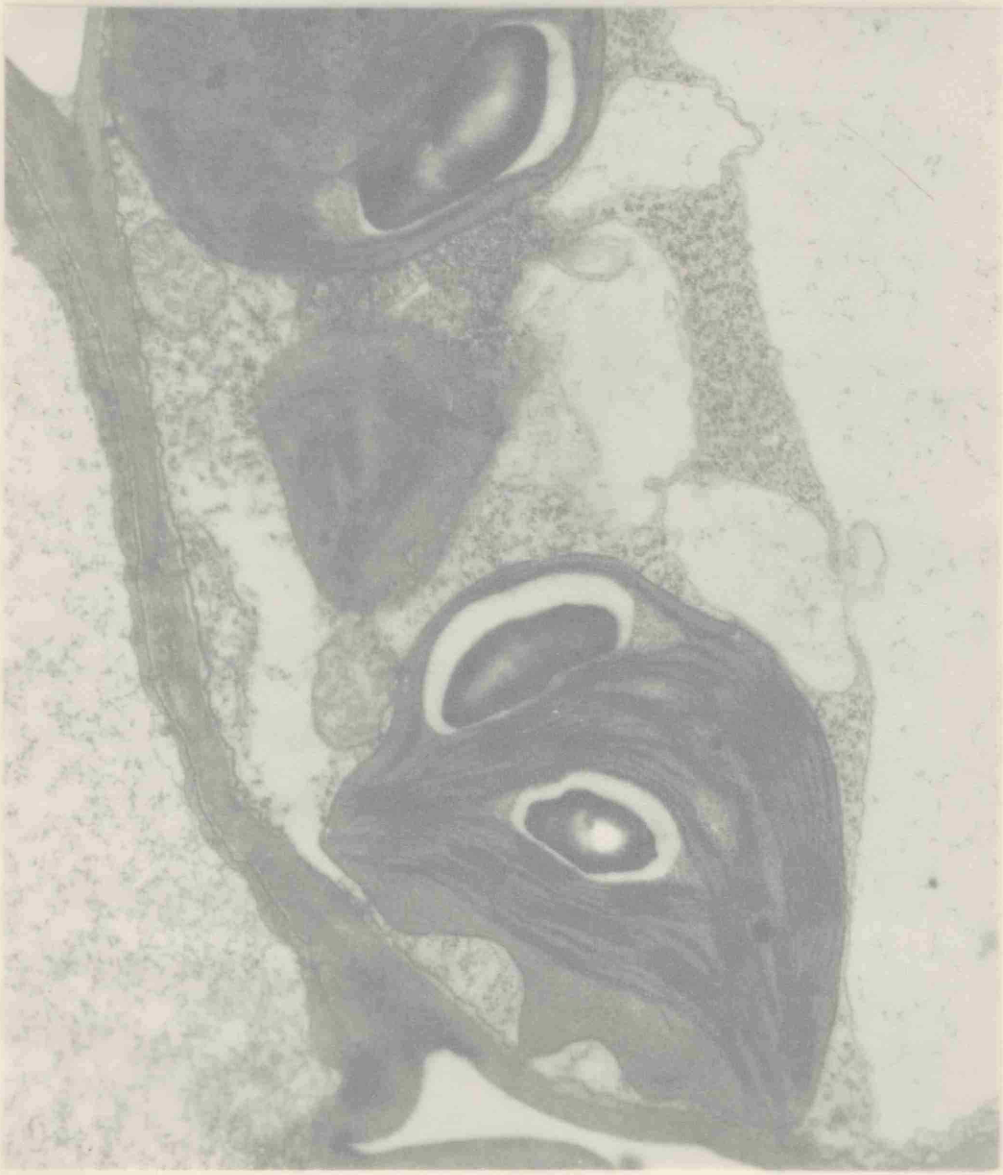


0.5μ

Note: tonoplast swelling away from outer chloroplast membrane; internally chloroplast similar to control.

0.5μ.

Plate 6. Appearance of vacuoles within the cytoplasm after 8 hours paraquat treatment.

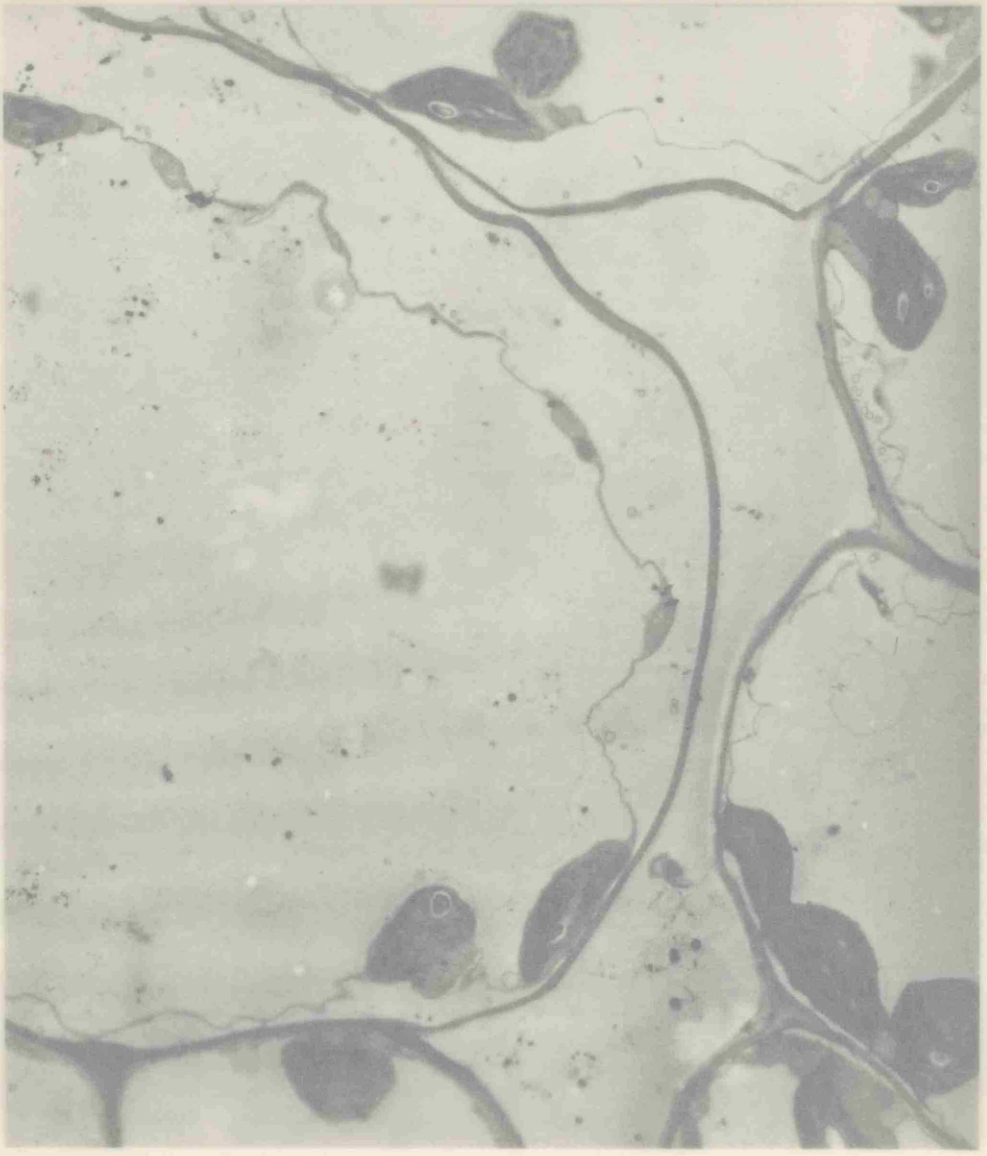


1μ.

Note: loss of some cytoplasm integrity; mitochondria present; change in chloroplast shape.

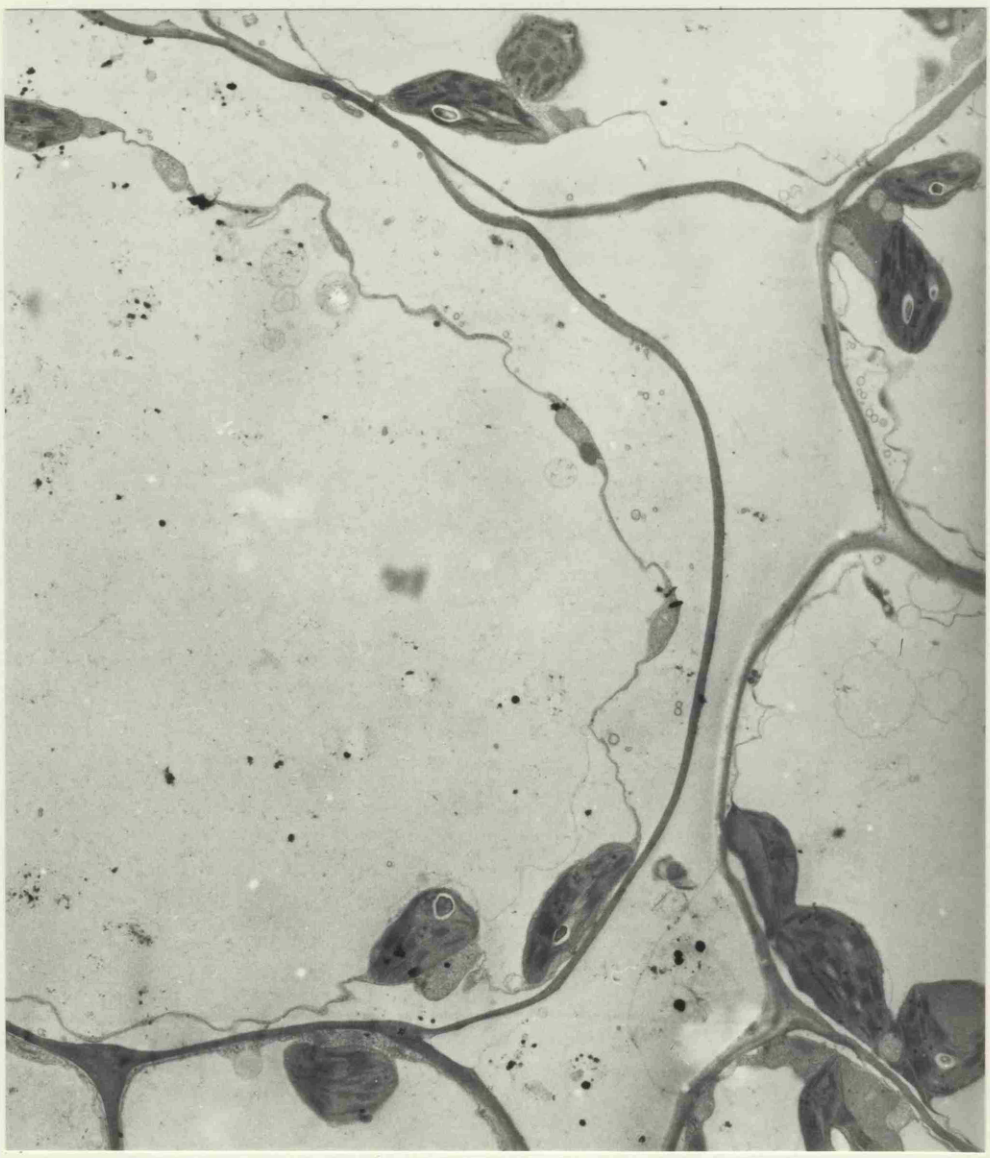
1μ.

Plate 7. Appearance of flax cotyledon leaf cell after 6 hours paraquat.



15μ.

Note: plasmalemma lying away from the cell wall; tonoplast no longer close to the chloroplast outer membrane.



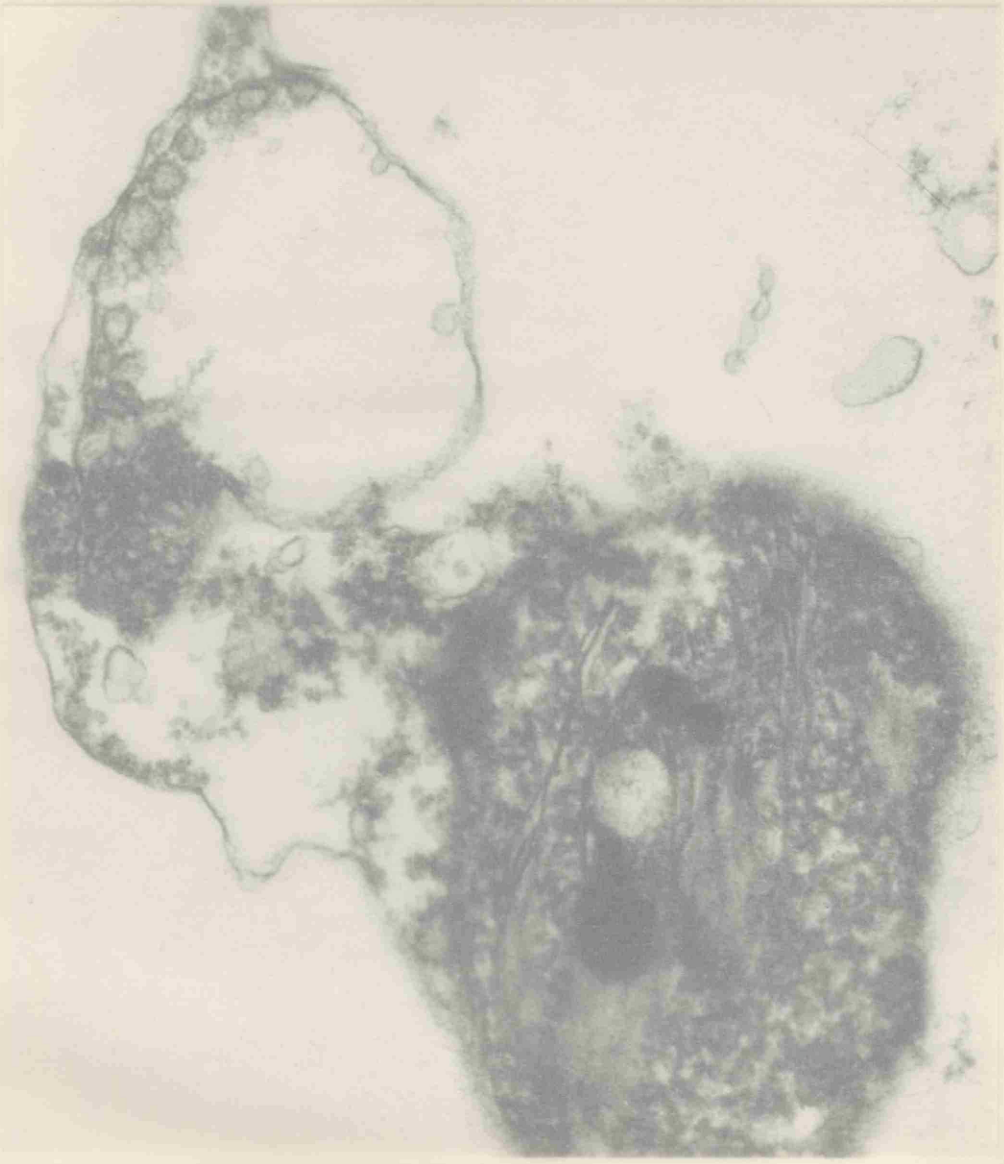
15μ.

Plate 8. Loss of tonoplast integrity after 6 hours paraquat.

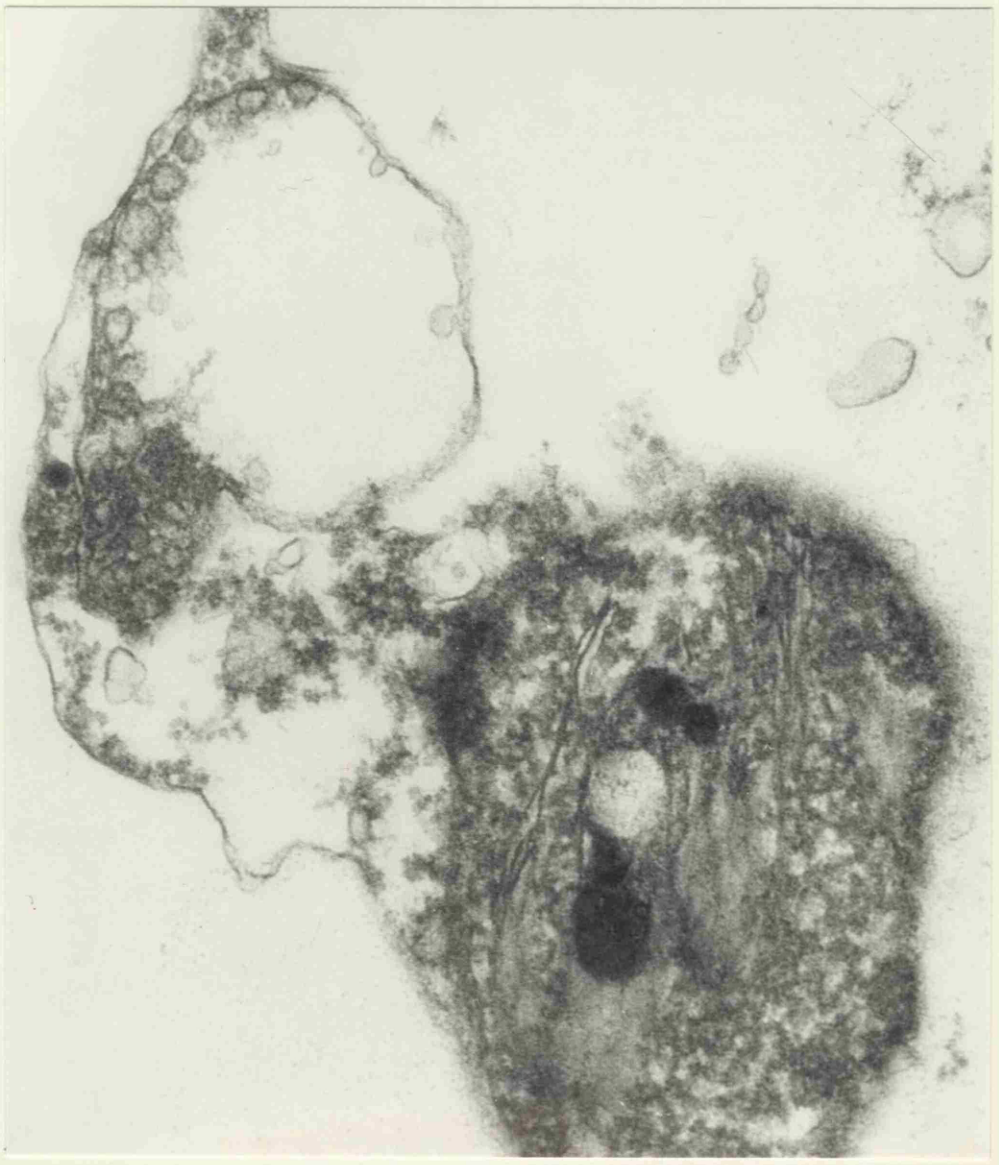
1μ.

1μ.

Plate 9. Effect of 9 hours paraquat on mitochondrion of flax cotyledon leaf cell.

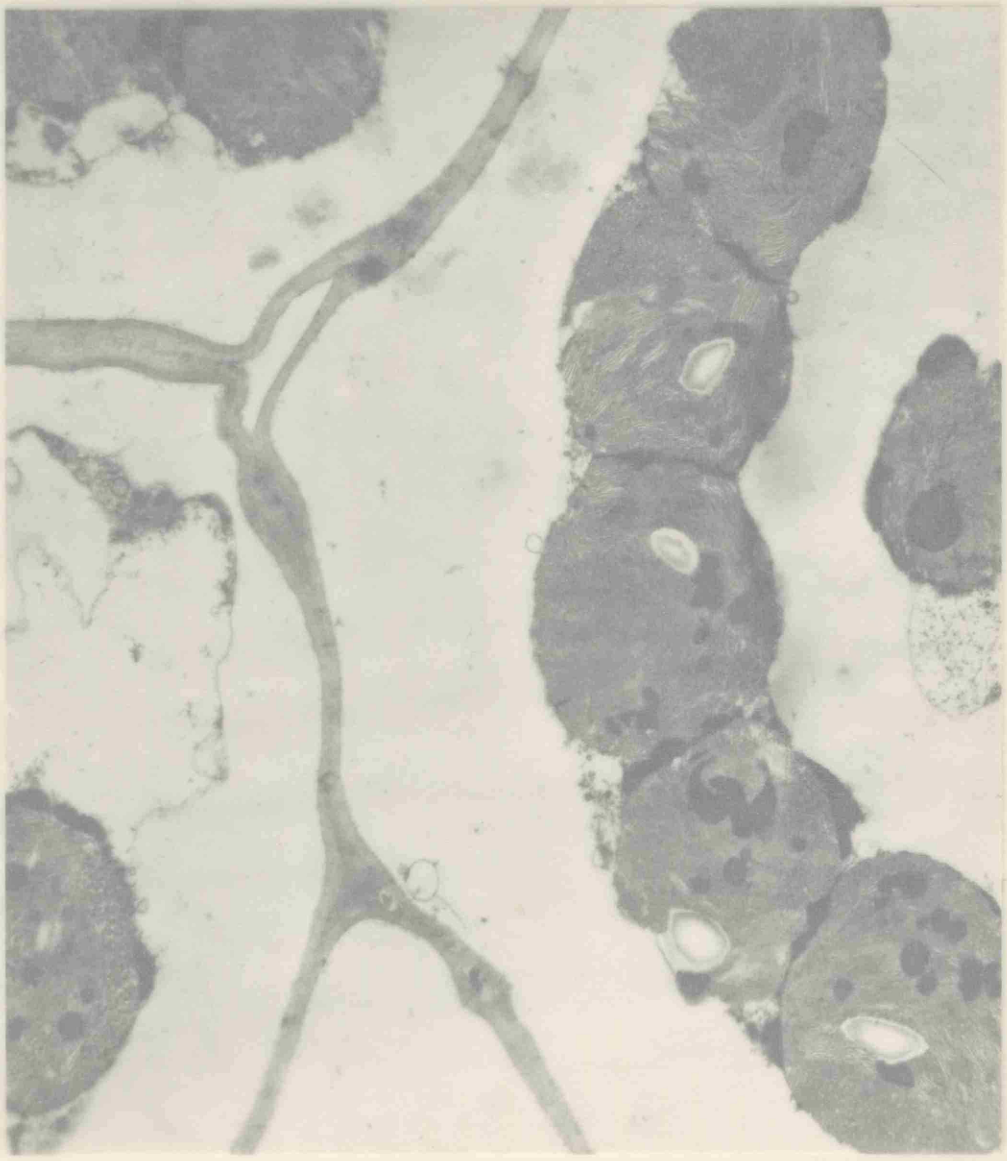


Note: mitochondrion shows considerable swelling; mitchondria are not seen after this time of paraquat treatment, possibly the swelling results in bursting.



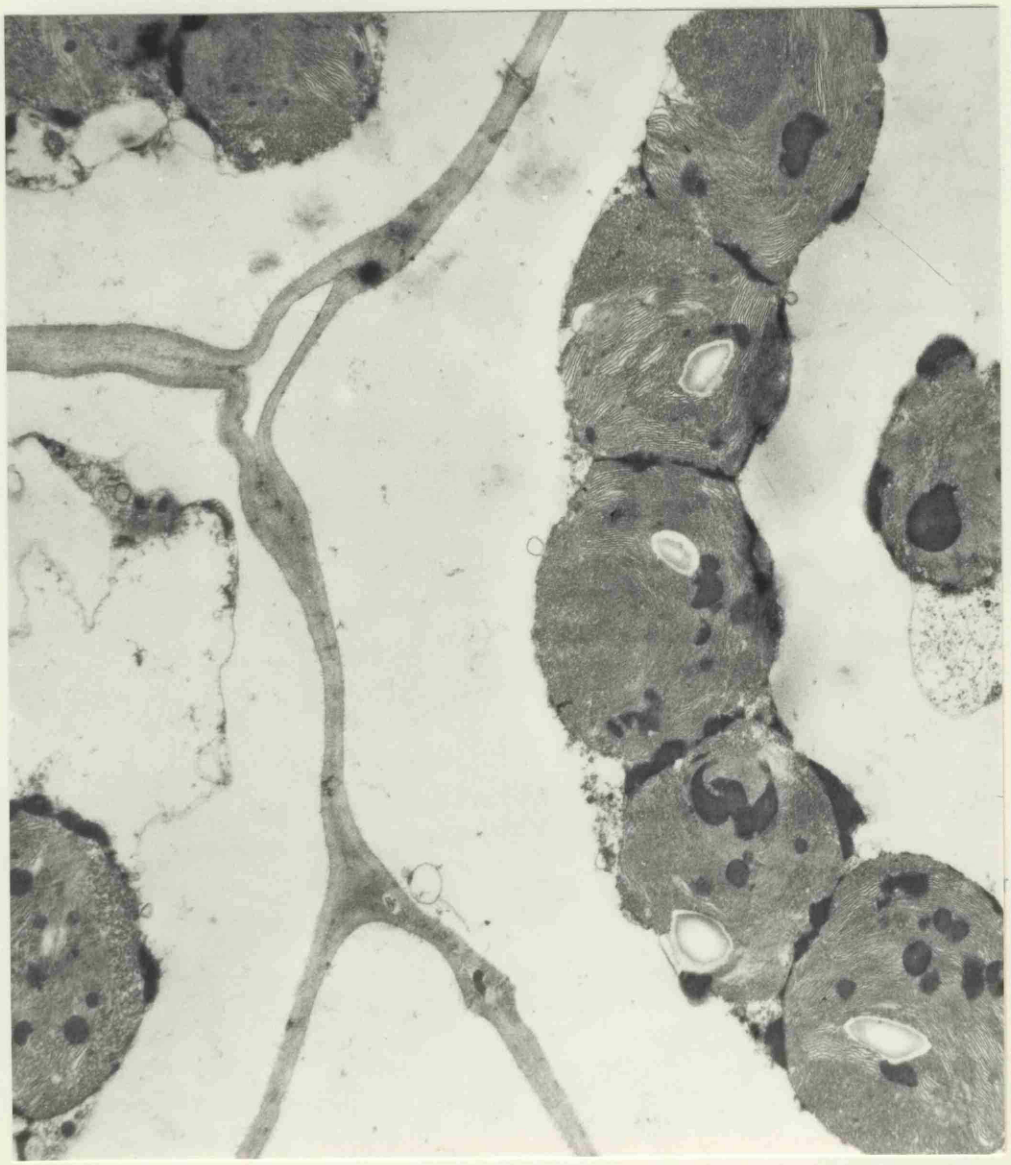
۱μ.

Plate 10. Appearance after 18 hours paraquat treatment.



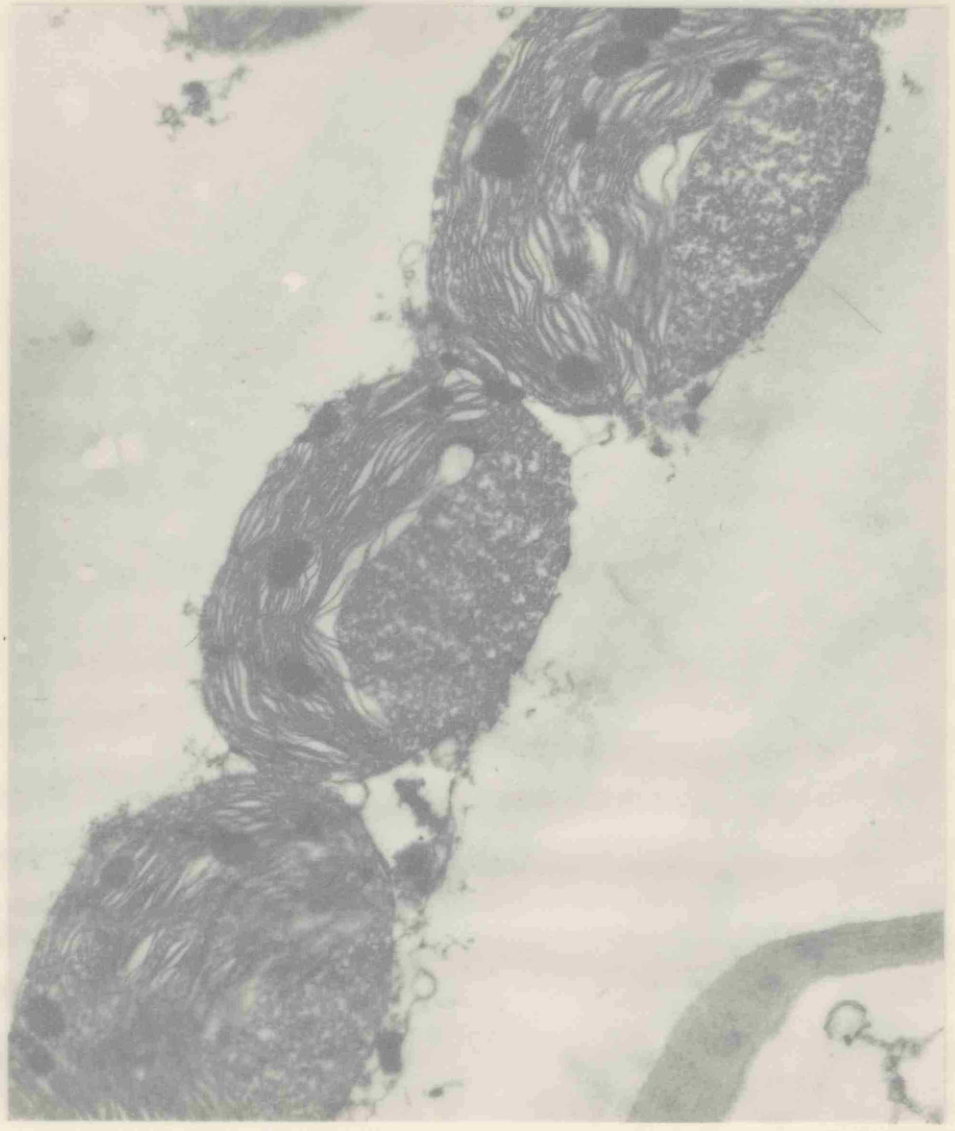
2μ

Note: accumulation of osmiophilic globules within the chloroplasts.



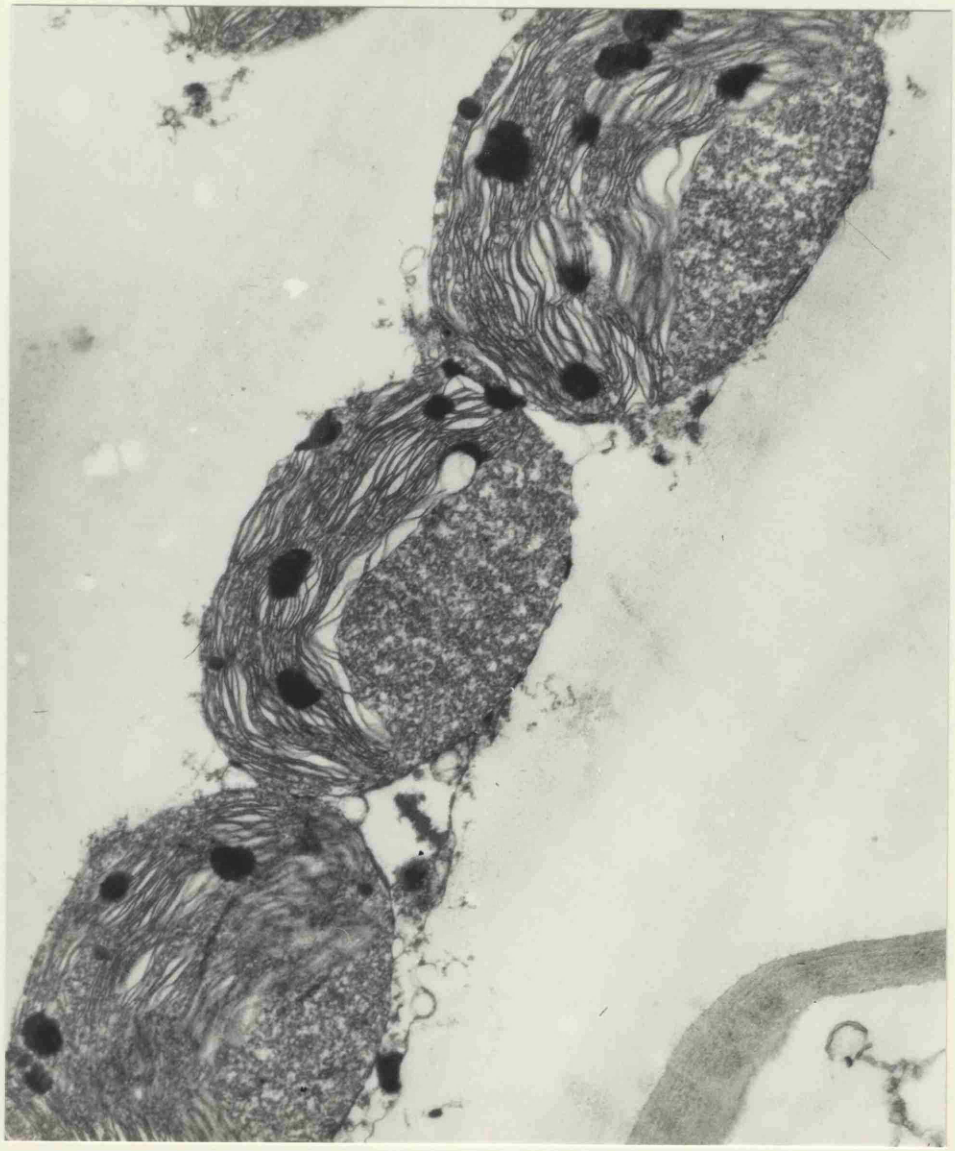
2μ.

Plate 11. Appearance after 18 hours paraquat treatment.



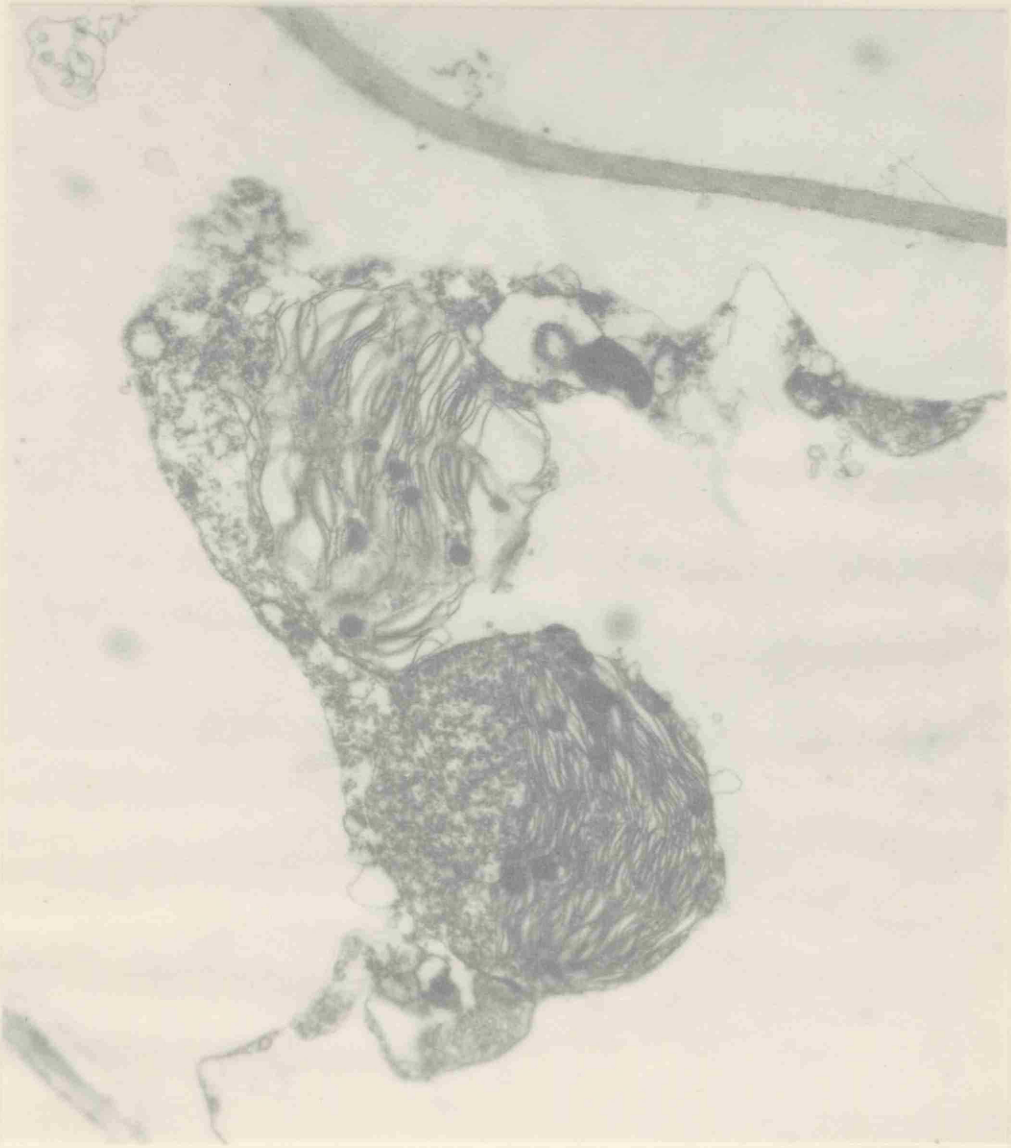
1 μm.

Note: loss of grana organisation; accumulation of osmiophilic globules; change in appearance of stroma.



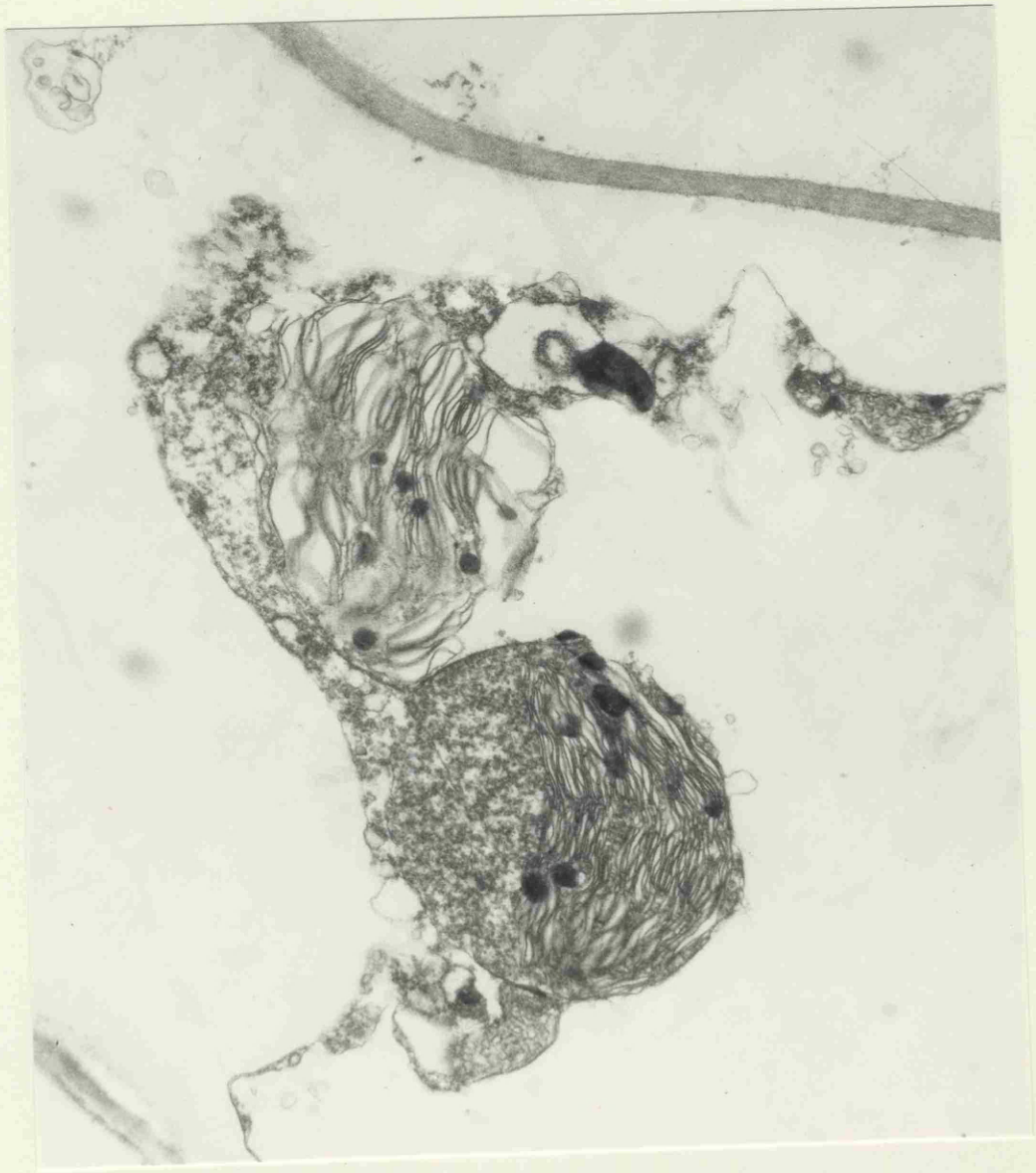
1 μ.

Plate 12. Appearance after 24 hours paraquat.



2μ.

Note: disintegration of chloroplasts with breakdown of stroma and lamellae.



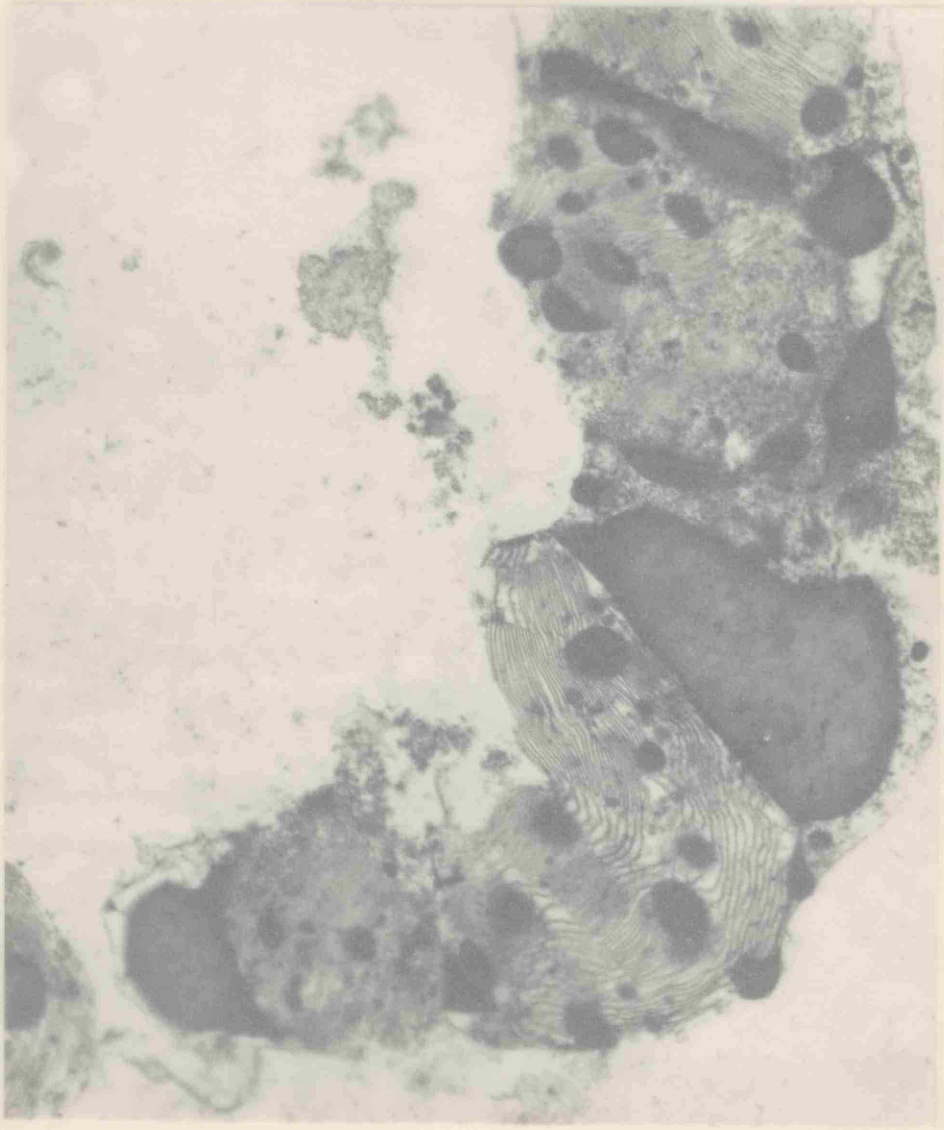
2μ.

Plate 13. Appearance after 30 hours paraquat.

Note: chloroplast stroma broken down or dispersed; change in appearance of nucleus (top right) as compared with Plate 3.

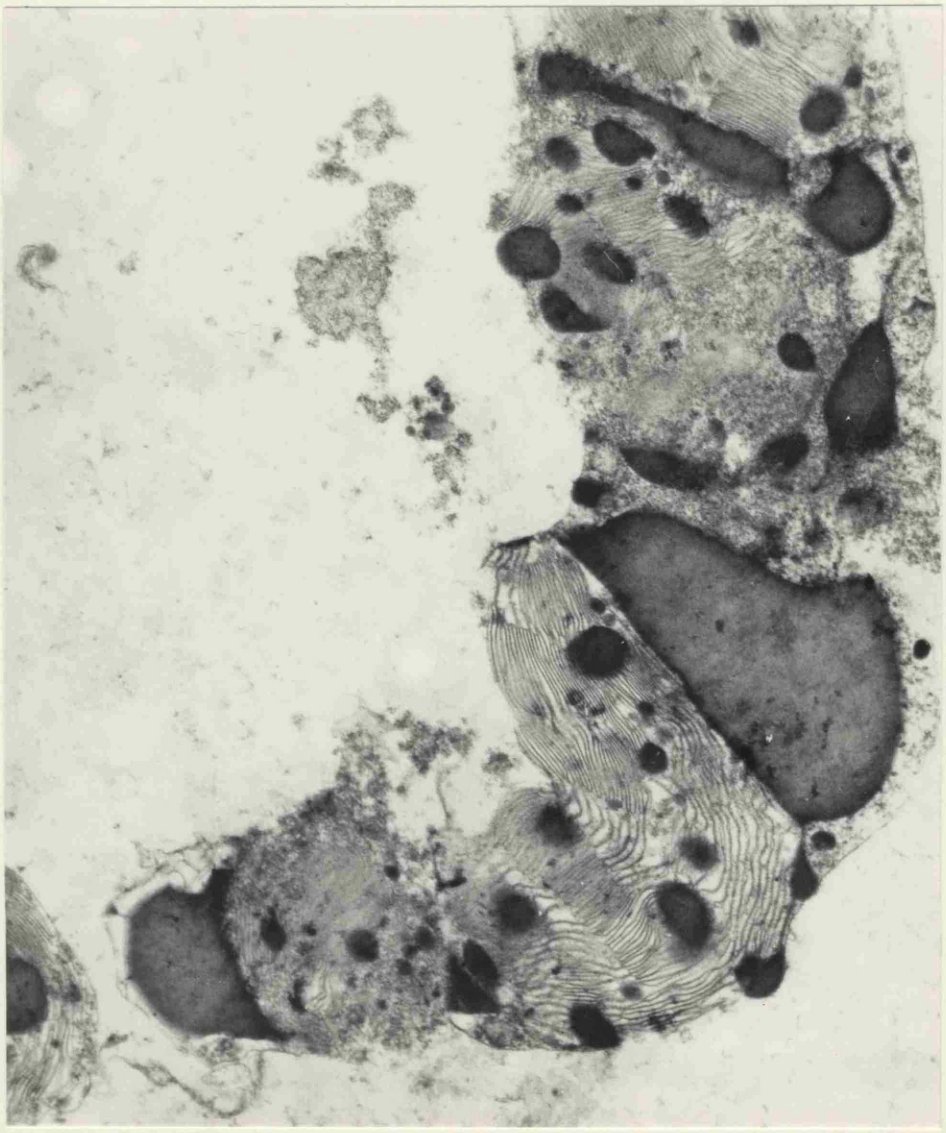
2μ.

Plate 14. Appearance after 30 hours paraquat.



1μ.

Note: massive accumulation of osmiophilic globules; stroma broken down or dispersed.



1 μm.

These results agree with other data which indicates a rupture of the tonoplast at about this time.

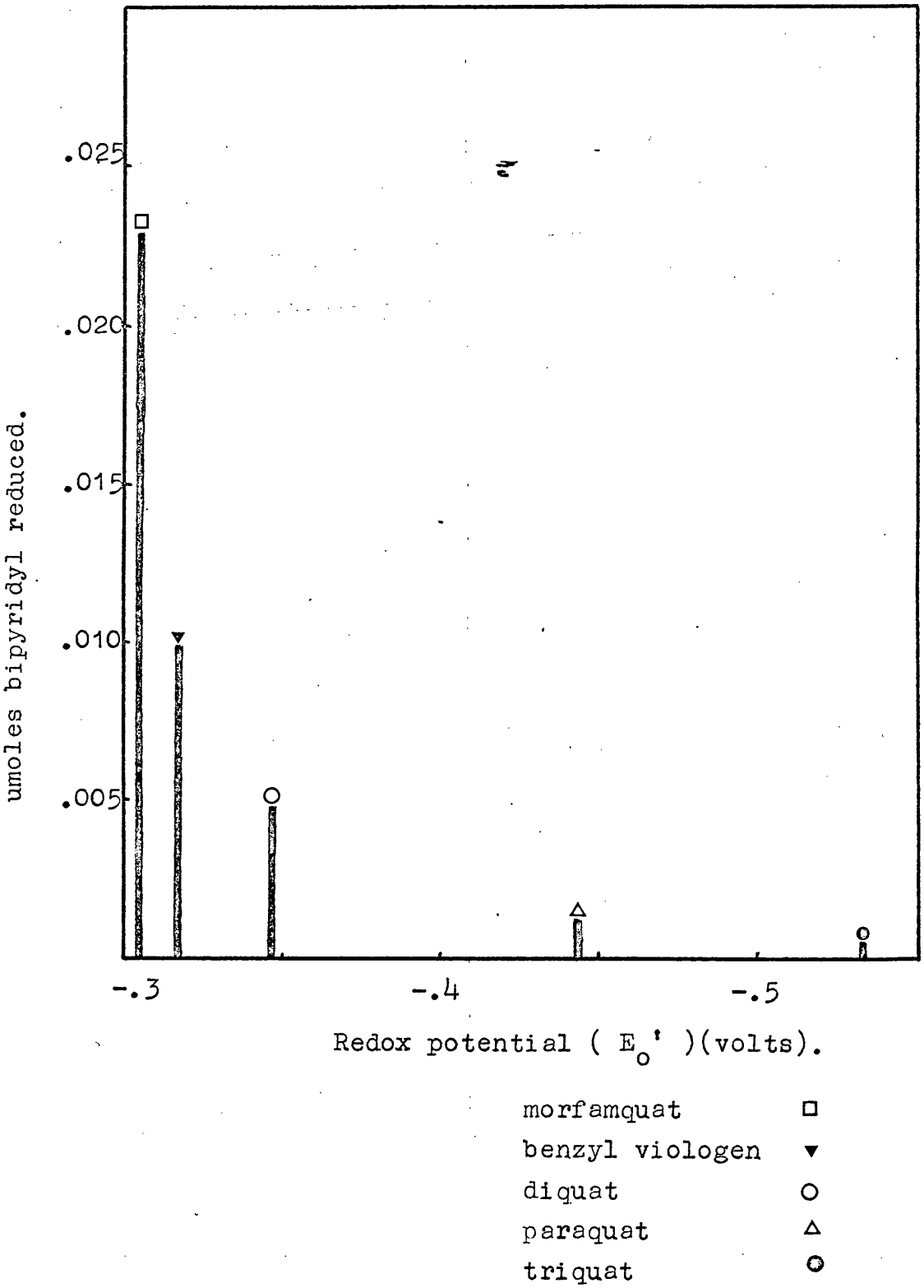
11. Some interactions of some bipyridyls with photosynthetic electron transport

As stated above, it is considered that the primary toxic product resulting from the action of paraquat and diquat is the result of an interaction of the herbicide molecule with the reducing potential provided by photosynthetic electron transport. Zweig, Shavit and Avron (1965) studied the inhibition of NADP reduction during the interaction of diquat with photosynthetic electron transport and Kok, Rurainski and Owens (1965) followed the photoreduction of a range of bipyridyls.

Comparative studies were carried out with diquat, paraquat, morfamquat, benzyl viologen and triquat. The photoreduction and subsequent re-oxidation of these compounds was measured and also their inhibition of NADP photoreduction.

Figure 2[illegible] shows the degree of reduction of these bipyridyls when illuminated with pea chloroplasts under anaerobic conditions. CMU was used to inhibit the normal oxygen evolution during photosynthetic electron transport and the chloroplasts were supplied with ascorbate as a source of reducing potential. Care was taken when using ascorbate that the concentrations of this and the bipyridyls were not at such a level to support auto-oxidation of the ascorbate (Orr, 1966). The quantitative reductions of the various bipyridyls were compared by

Figure 29. Comparative reduction of five bipyridyls by pea chloroplasts.



supplying a standard amount of 'photosynthetic reducing power' (i.e. a constant amount of chloroplast preparation illuminated for a standard time) and measuring the amount of reduction. In these experiments the bipyridyls were in excess in order to ensure maximum reduction.

A further series of experiments involved the determination of the rates of oxygen uptake mediated by the bipyridyls. Figure ~~29~~ 30 shows the increase in oxygen uptake with increasing concentration of the five bipyridyls. At lower concentrations there was no difference between the rates of oxygen uptake by the five compounds, but at higher concentrations (i.e. above 10^{-6}M) it appeared that the rate of oxygen uptake was related to the E_o' of the compounds. The bipyridyl concentration at which oxygen uptake and E_o' were related was dependent on chlorophyll concentration.

The inhibition of photosynthetic NADP reduction by the bipyridyls was found to be dependent on the concentration of the bipyridyl, but not the E_o', and the concentration of chlorophyll. Figure ~~30~~ 31 shows comparative inhibitions of NADP reduction by the five bipyridyls with a standard chlorophyll concentration.

The concentration of bipyridyl which completely inhibited NADP reduction was found to be of the same order as the concentration at which photo-oxidation of ascorbate became related to bipyridyl E_o'. Calculations based on the results of bipyridyl concentration and the amount of chlorophyll indicate that there were approximately 150 moles chlorophyll per mole bipyridyl when NADP reduction was completely

Figure 30. Oxygen uptake mediated by five bipyridyls.

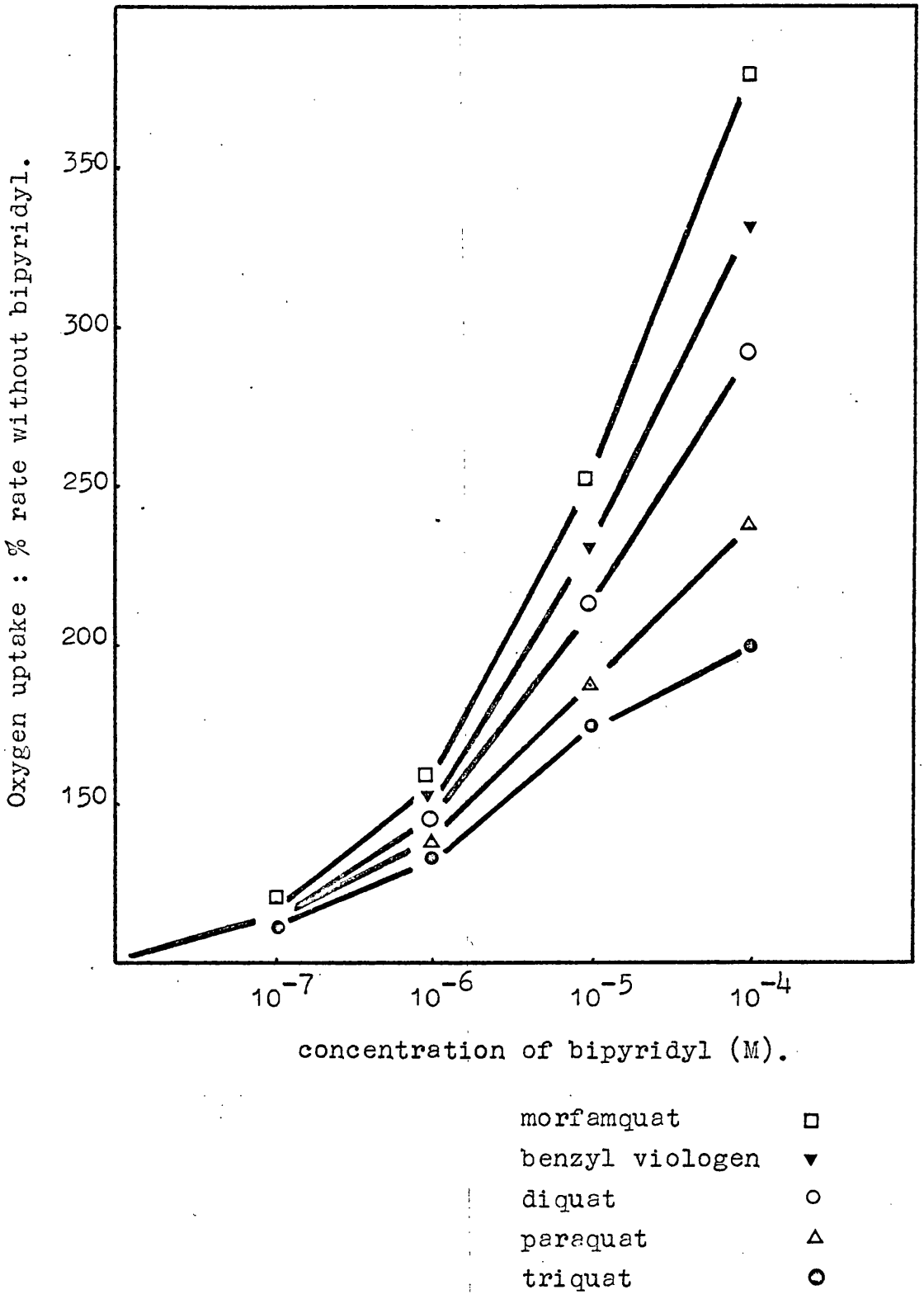
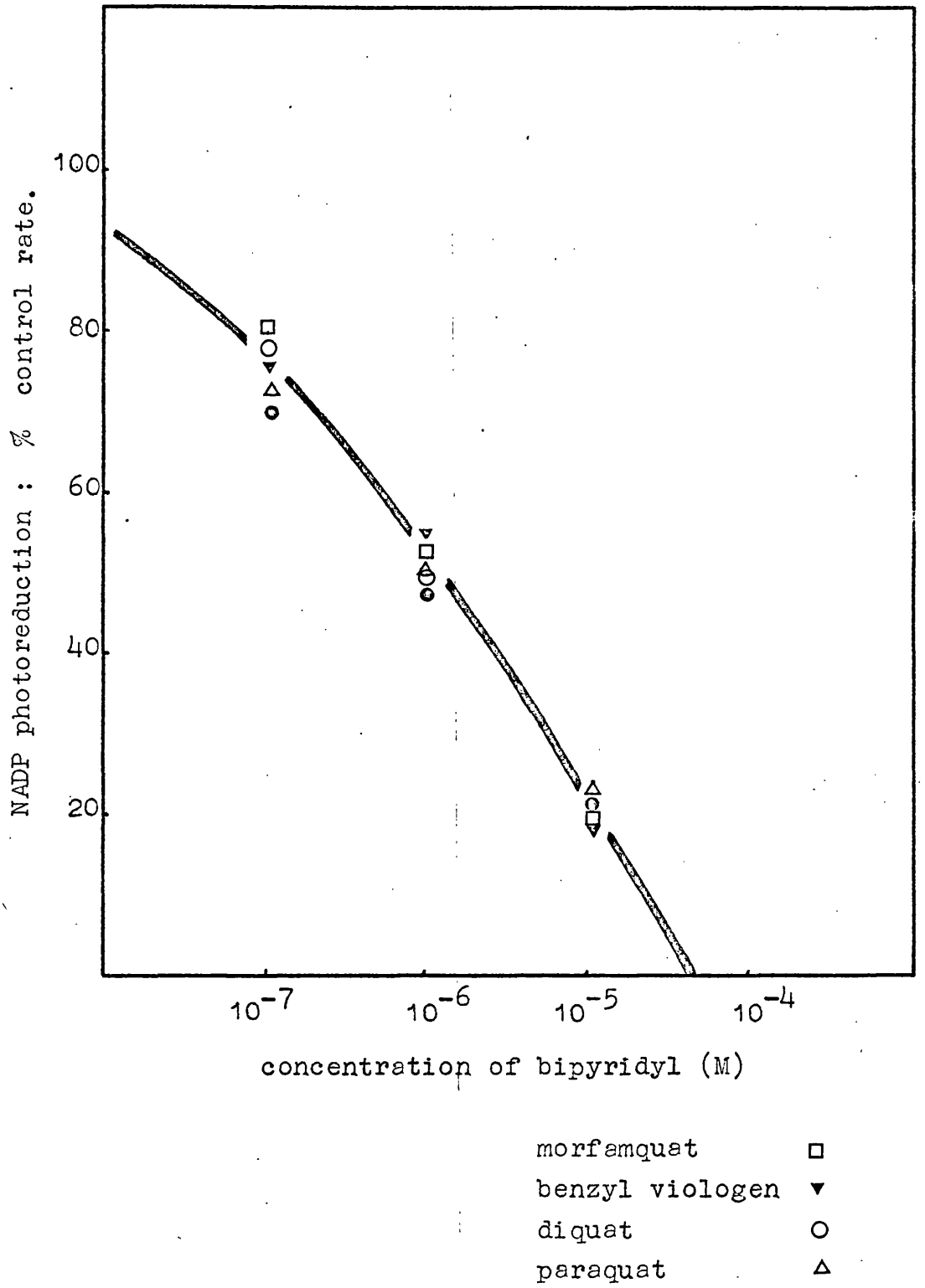


Figure 31. % inhibition of NADP photoreduction by five bipyridyls.

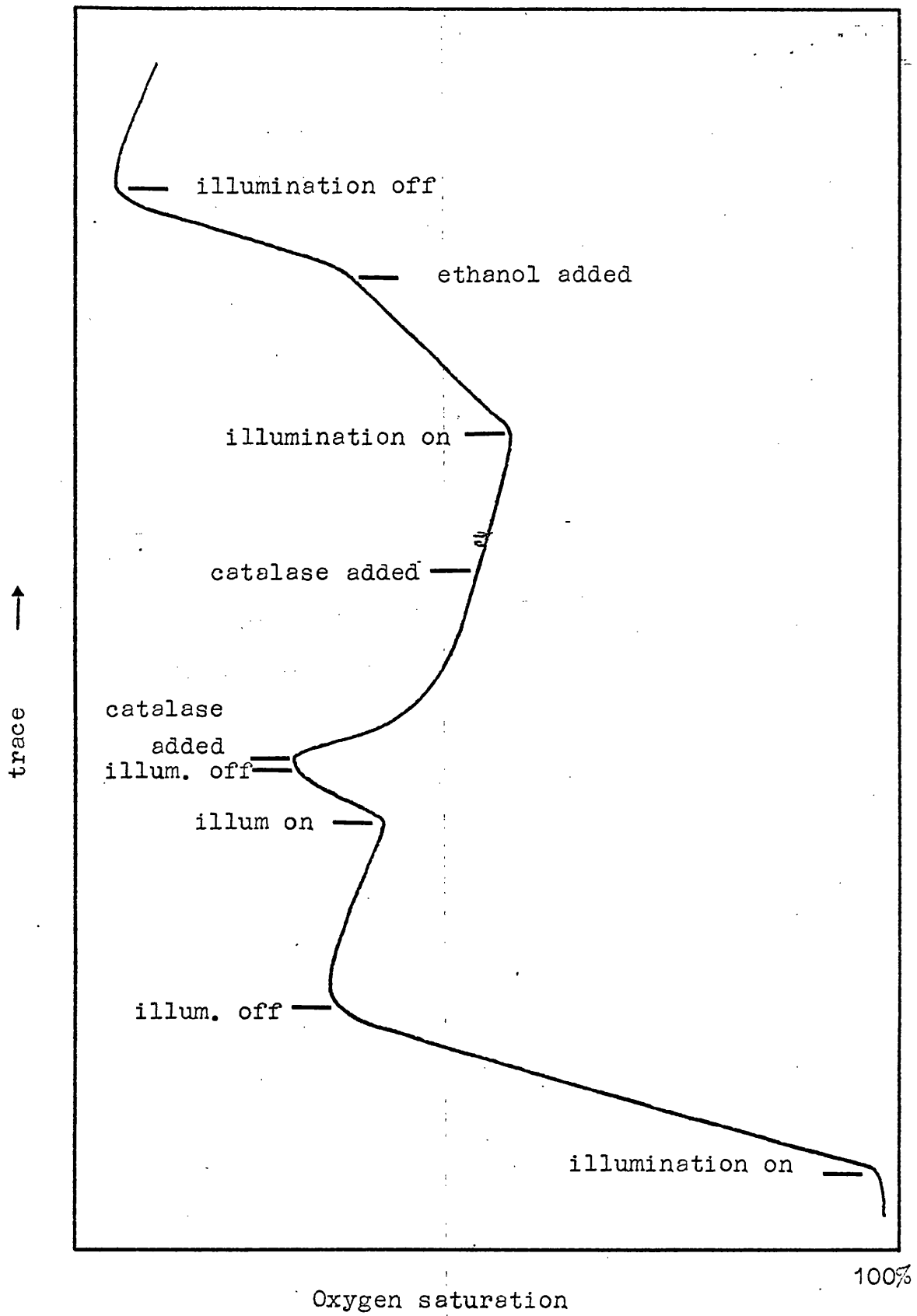


inhibited and ascorbate photo-oxidation was related to bipyridyl E_o'. The figure of 150 moles chlorophyll per mole bipyridyl agrees very closely with the figure given by Baldwin, Clarke and Wilson (1968) relating to the amounts of paraquat they found in chloroplasts isolated from herbicide treated leaves.

12. Production of hydrogen peroxide by illuminated chloroplasts with paraquat

Evidence of the production of hydrogen peroxide was obtained with chloroplasts illuminated with paraquat. Figure 32 shows an oxygen electrode trace of an experiment in which heated chloroplasts (55°C for 10 minutes) photo-oxidised ascorbate with paraquat. The heat treatment inactivated endogenous catalase activity associated with the chloroplast suspension. After a period of illumination there was a slight rise in oxygen tension during a dark period. Following the next period of illumination there was a further dark period at the beginning of which catalase was added to the cuvette. There was an immediate rise in oxygen tension, indicating the release of oxygen by the action of the catalase, followed by a slow rise as before. Further catalase was added without effect. The suspension was re-illuminated and found to have a reduced rate of oxygen uptake, but this was restored to the original rate by the addition of ethanol. The reduced rate before addition of ethanol could be explained by the effect of added catalase, but after the addition of ethanol there would have been no oxygen release as this acts as a trap, with catalase and hydrogen peroxide, producing

Figure 32. Oxygen electrode trace showing hydrogen peroxide production by pea chloroplasts with paraquat.



acetaldehyde.

It is of interest that the oxygen evolution resulting from the addition of the catalase did not account for all of the oxygen taken up during the periods of illumination. This would indicate that some of the oxygen taken up had already been bound, possibly in the peroxidation of membrane components of the chloroplast suspension within the cuvette.

13. Catalase distribution in flax cotyledon leaves

It had been reported that catalase activity was associated with the chloroplasts (Neish, 1939) and it might have been expected that any peroxide produced as a result of the interaction of paraquat with photosynthetic electron transport would be rapidly broken down.

Experiments on the distribution of catalase within flax cotyledon leaf cells showed that only 3-5% of the total catalase activity was associated with the isolated chloroplasts. The isolated chloroplasts were washed once to remove adsorbed catalase from the cytoplasm. It was thought that one washing would not remove catalase from within the chloroplast, and the activity associated with the chloroplasts remained con stant after three washings.

Gregory (1968) found a similar catalase distribution in spinach with only 5-10% associated with the chloroplasts.

D I S C U S S I O N

The discussion of the results given above will be developed in two sections. The first deals with the sequential effects of the herbicides paraquat and diquat on flax cotyledon leaves and the second deals with the interaction of the bipyridyls, morfamquat, benzyl viologen, diquat, paraquat and triquat with photosynthetic electron transport.

The first indication of the effects of diquat and paraquat on flax cotyledon leaves was an inhibition of the normal photosynthetic carbon dioxide fixation. This was accompanied by a slight rise in carbon dioxide evolution, possibly by respiration, and this was soon followed by alterations of the cell membranes. The electron micrographs of comparably treated cotyledon leaves showed, at this stage, a change in the tonoplast, and at the same time it was found that the cotyledon leaf cells were losing their ability to maintain potassium ions within the vauole. It has been reported that as much as 98% of the cell potassium is held within the vacuole with a slow rate of exchange (Diamond and Solomon, 1959). It would seem that the sudden and rapid efflux of potassium ions from the treated leaf cells indicated an alteration of the tonoplast.

Merkle, Leinweber and Bovey (1965) reported a similar phenomenon soon after the beginning of the treatment of bean, honey mesquite and honeysuckle with paraquat. They measured the resistance of the solution on which the leaves were treated and deduced that a

reduction in the resistance was due to a leakage of cell components which had been brought about by effects on membrane permeability. They reported that a similar effect was obtained in the light both with and without oxygen. However they do not appeart to have inhibited photosynthetic oxygen evolution, and therefore the system claimed to be anaerobic was probably not so.

It is generally accepted that light and oxygen are essential for the rapid kill by the bipyridyls (Mees, 1960; Akhavein and Linscott, 1968). The report of Merkle, Leinweber and Bovey (1965) and a paper by Baur, Bovey, Baur and El-Seify (1969) of electron micrographs showing similar effects in the light and dark with paraquat treated honey mesquite contradict this, but practical details in both papers are questioned.

The rise in carbon dioxide given off during paraquat treatment might be expected as a consequence of a paraquat interaction with mitochondrial electron transport. The upsetting of the normal control mechanism of electron flow to NAD reduction would show as a change of carbon dioxide evolution in the dark.

The leakage of potassium ions from the cotyledon leaf cells had begun after 6 hours treatment with paraquat, at which time the electron micrographs showed changes in the tonoplast of the upper cells of the spongy mesophyll. The capacity for photosynthetic carbon dioxide uptake was completely inhibited by $4\frac{1}{2}$ hours, a time which corresponds with the estimates of the time taken for the herbicide to reach its site of action throughout the cotyledon leaf. It should be noted that the times for complete inhibition of the various processes within the cell are extrapolations from the plots of the decrease of activity.

The times are only accurate within the variations of the time needed to carry out the particular assay.

Baur, Bovey, Baur and El-Seify (1969) reported the effects of paraquat on the ultrastructure of honey mesquite mesophyll cells. They noted that the first visible change induced by the herbicide was a rapid disintegration of the plasmalemma, followed by a rupturing of the chloroplast membranes and loss of chloroplast turgor. They also noted that the changes that occurred in plants treated in the light were similar to those in plants treated and sampled in the dark. However, they commented in their discussion that the dose of paraquat applied might have been excessive, and they also used potassium permanganate as a fixative. Potassium permanganate is an oxidative fixative and its usefulness is largely confined to preserving lipoprotein complexes which occur in the membrane systems of cells. It is not always uniform in its effects and most investigators are inclined to place less reliance on it as compared with osmium tetroxide (Pease, 1964).

At the same time as the beginning of the efflux of potassium ions from the cotyledon leaf cells, there was a small rise in the level of malondialdehyde within the cotyledon leaves. Malondialdehyde is a breakdown product of the peroxidation of unsaturated fatty acids (Patton and Kurtz, 1951) and it is thought that in this case the rise in the level of this compound might be associated with the breakdown of membrane lipids.

It was also found that after 6 hours illumination with paraquat there was a drop in the Photosystem II activity of chloroplasts isolated

from treated cotyledon leaves. While there was a drop in the rate at which Photosystem II activity was being lost if the cotyledon leaves were placed in the dark, it is significant that this was slower than in the light. As the period of illumination was increased in length the effectiveness of the dark incubation, in reducing the rate of loss of activity, decreased and after some 15 hours treatment in the light there was an equal decline subsequently in both the light and dark. The lost activity during the light incubation was never regained, indicating that from the early stages the degenerative course of events was irreversible. This is in marked contrast to senescence, where the processes can be well advanced and yet still reversed if the condition of the plant is altered. For example, Ljubesic (1968) reported that cotyledon leaves regreened if the apical bud was removed at a stage when the cotyledons were becoming yellow. Sveshnikova, Kulaeva and Bolyakina (1966) reported that during the ageing of tobacco leaves the chloroplasts in old yellow leaves contained a large number of osmiophilic globules and the remains of lamellae. Under the action of the senescence inhibitor 6-benzyl-aminopurine there was a construction of new lamellae in such chloroplasts and later the formation of new grana. This was accompanied by the gradual disappearance of the osmiophilic inclusions. There is however a stage after which the course of senescence cannot be reversed. It is possible that this point in the sequence of events is related to tonoplast integrity.

Shaw and Manocha (1965) considered that the rupture of the tonoplast was a major contributing feature in the course of senescence. A direct result of tonoplast rupture would be a loss of cell organisation and osmotic balance with a consequent loss of metabolic control, and the liberation of toxic products often stored within the vacuole could indirectly result in further deterioration.

The involvement of some process other than the direct effect of the bipyridyls via photosynthetic electron transport is indicated by the continued cellular disintegration after a time when it is assumed that there was no further effective photosynthetic electron transport within the cell.

The results have shown that within 18 - 30 hours (depending on the temperature) there was a loss of Photosystem II activity in chloroplasts isolated from herbicide treated cotyledon leaves. After this time it is assumed that there was no further production of a toxic product (possibly hydrogen peroxide) via the bipyridyls and photosynthetic electron transport. Theoretically it might be possible for a small amount of electron transport to occur after this time. It was found that chloroplasts isolated from paraquat treated cotyledon leaves lost the requirement for an intermediate when using an electron source such as ascorbate for in vitro maintenance of Photosystem I activity. As ascorbate is present within the cell this might be able to link directly with Photosystem I to support some photosynthetic electron transport. This will be mentioned later in the discussion.

If chlorophyll breakdown is used as the indicator of paraquat action then a different picture is obtained to that using Photosystem II activity. Although there was no difference between subsequent light or dark treatment after 15 hours with regards to Photosystem II activity fall off, there was a significant effect on chlorophyll degradation. Even after 24 hours there was a reduction in the rate of chlorophyll breakdown if the cotyledon leaves were placed in the dark. Of the parameters measured it was only the pigments that were dependent upon light for a higher rate of breakdown at this stage. It was reported that chlorophyll photo-oxidation may be inhibited by the presence of carotenoids in vivo (Koski and Smith, 1951; Rabinowitch, 1956) as there is a rapid energy transfer from the chlorophyll triplet state to carotenoids (Kranowski, Drozdowa and Pashina, 1960; Fujimori and Taula, 1966). During the action of paraquat and diquat there was a breakdown of the carotenoid pigments and it is possible that the breakdown of the chlorophyll was brought about by photo-oxidation and was therefore dependent on light even after the cessation of photo-synthetic electron transport and the consequent production of a toxic product.

The major breakdown of the pigments occurred after about 20 hours treatment. Reference to the electron micrographs showed that by this stage the cellular disorganisation was well advanced and there was some breakdown of the organised structure within the

chloroplast. By 30 hours the electron micrographs showed that the chloroplasts were completely disorganised. If all cells were in that state it would have seemed unlikely that isolated chloroplasts would still have shown some capacity for ascorbate photo-oxidation. However, it should be noted that the micrographs show the most advanced stages at a particular time of herbicide treatment. It is reported that Photosystem I is far more stable than Photosystem II (Vernon and Zaugg, 1960; Davenport and Dodge, 1969) and electron micrographs of sonicated chloroplast preparations, still capable of ascorbate photo-oxidation, show that a very advanced state of lamella disruption is required before there is a complete loss of activity (Jacobi and Lehmann, 1969).

Also associated with the gradual deterioration of the chloroplast structure was a loss of a requirement for the DCIP link for Photosystem I ascorbate photo-oxidation. Other treatments such as ageing, heating, sonication and detergent treatment of chloroplast preparations all remove the requirement for the couple, and it may well be that the lack of this requirement is an indicator of a degree of chloroplast structural damage (Davenport and Dodge, 1969). In all of these cases as with the treatment with diquat and paraquat, continued treatment leads to a complete loss of ascorbate photo-oxidation activity.

The more rapid breakdown of chlorophyll a than of chlorophyll b found during the course of the action of diquat and paraquat was similar to the pattern seen during other processes of

green plant breakdown (e.g. food processing (Tan and Francis, 1962), extraction of pigments (Aronoff, 1943; Strain, 1958) and senescence (Wolf, 1956; Bacon and Holden, 1967).

A most apparent feature of the change in appearance of the chloroplasts during the course of the treatment with diquat and paraquat was the massive accumulation of osmiophilic globules, which commenced after some 12 hours.

Ikeda and Ueda (1964) studied the structural changes in chloroplasts of senescing leaves of Elodea and found that as the thylakoids broke down large osmiophilic globules appeared in the stroma. A similar breakdown was reported to occur in the senescing leaves of Phaseolus (Barton, 1966) and was also shown by Shaw and Manocha (1965) in their studies with wheat, although here the accumulation was not so great. In both Phaseolus and wheat these changes were accompanied by a loss of ribosomes and endoplasmic reticulum, and also by the degradation of other organelles.

Ikeda and Ueda (1964) considered that the globules could be an accumulation of the chloroplast membrane breakdown products. However, in cucumber (Butler, 1967) and also in other tissues (von Wettstein, 1959) the globules first appeared during thylakoid formation. Greenwood, Leech and Williams (1963) working with globules from mature chloroplasts from Vicia faba and Bailey and Whyborn (1963) with spinach found that the globules contained a mixture of lipids not all of which were chloroplast membrane components. The evidence would thus indicate that although the

globules in senescent chloroplasts may in part be membrane breakdown products or precursors, they probably also represent a general store of insoluble lipid material not necessarily connected with membrane formation or breakdown.

Not only was the disintegration of the ordered lamellar system and the accumulation of osmiophilic globules an obvious feature during the progress of the action of diquat and paraquat, but the stroma also showed considerable change after the rupture of the chloroplast double membrane. The granular appearance of the stroma is considered to be a consequence of the presence of protein and nucleic acid particles and it could well be that the change in appearance was due to changes of these compounds.

The breakdown of the major membrane lipids, monogalactosyl and digalactosyl diglyceride and the phospholipids was seen as a major feature of the overall changes in the pattern of lipid composition. Wintermans (1960), in a comparison of phosphotides and glycolipids of yellow and green leaves and of chloroplasts and whole leaves, concluded that chloroplasts of green leaves were particularly rich in phosphotidyl glycerol and mono- and di-galactosyl diglyceride. Sastry and Kates (1964) and Helmsing (1969) identified galactolipase in plant tissue, although they disagreed as to whether there were specific enzymes for the breakdown of mono- and di- galactosyl diglyceride (Sastry and Kates) or one enzyme which attacked both (Helsing). Both groups found that they could not detect lyso compounds after the action of galactolipase and concluded that this was apparently due to the fact that the

formation of the galactosyl glycerols had a higher reaction rate than the deacylation reaction of the galactolipids to the lyso compounds. The reaction products that they found were galactosyl glycerol and fatty acids.

Following the initiation of the kill by diquat and paraquat there was an increase in a mono-galactosyl diglyceride which had a fatty acid profile similar to that of the normal digalactosyl diglyceride. Later there was an increase in mono-glycerides and diglycerides with fatty acid profiles which indicated that they might have originated from the galactosyl diglycerides and phosphotidyl ethanolamine. These indications of changes in membrane structure, however, did not occur until after some 24 - 30 hours treatment with the herbicides.

Earlier indications of changes in membrane structure were found in the increase of potassium ion efflux from the cotyledon leaf cells which began after 4 hours herbicide treatment. This would have occurred with the breakdown of tonoplast integrity, but as the tonoplast represents only a small portion of the total membrane within the cell a noticeable change in the overall composition of lipids would not be expected.

However, although the change in the total level of lipid was not measurable at this time, the accumulation of the lipid peroxidation product malondialdehyde was measurable and of significance as it occurred at the same time as the electron micrographs indicated a rupture of the tonoplast.

The major lipid changes indicated above suggest breakdown of the chloroplast internal membrane structure. Alterations in the lipid composition observable by thin layer chromatography of total lipid extracts began at about the same time as the electron micrographs showed the start of the breakdown of chloroplast structure.

Micrographs showing cellular breakdown correlated well with changes in levels of cell constituents and showed that the cells were losing material rapidly during the course of the kill by paraquat and diquat. The levels of total soluble protein and soluble carbohydrate dropped along with the fresh and dry weights. The pattern of these declining levels of cell constituents was similar to those found with senescing leaves (Lewington, Talbot and Simon, 1967), although it was noticeable that during the course of the action of paraquat and diquat there appeared to be no new synthesis of enzymes, as has been reported to occur during senescence (Kessler and Monselise, 1961; Hanson and Swanson, 1962; Lewington, Talbot and Simon, 1967).

A significant difference between the sequence of events found during the action of paraquat and diquat and that reported to occur during senescence was the time at which the leaves lost the ability to retain ions normally retained within the vacuole. The leakage of potassium ions from paraquat treated cotyledon leaves was one of the first indicators that the cells were being affected. With senescent flax cotyledon leaves the tonoplast

retained its ability to contain potassium ions until the sequence of senescence was more advanced. Eilam (1965) reported that increase in permeability was an early indication of senescence, although considerable amounts of potassium ions were retained within the cell until later.

Attention was drawn, in the introduction, to the theory that the vacuole may contain a number of hydrolytic enzymes as well as the various breakdown products which are deposited away from the cytoplasm. Dingle (1968), in a review of lysosomes of animal tissue, considered that it was likely that the control of cellular digestive function in both normal physiology and pathology might be largely dependent upon alteration of the lipoprotein and glycoproteins of the plasma membranes and the organelles. The ideas of Matile that the vacuole is, in effect, a lysosome appear to correlate with the evidence found in this series of investigations into the sequence of events during the action of paraquat and diquat.

Matile (1968) noted that there was a complement of hydrolytic enzymes (proteases, nucleases, carbohydrases, phosphatase and esterases) contained in the cell vacuole which could catalyse the breakdown of material released from the cytoplasm into the vacuole. The phenomenon of plant metabolite turnover, demonstrated to be a common feature of cells (Bidwell, Barr and Steward, 1964) is dependent on the intracellular digestion of macromolecules and portions of the cytoplasm. If there was an upset of this normally controlled system by the removal of the normal barriers

which physically separate digestion and synthesis, the cell would be open to considerable autolysis.

Barton (1966) suggested that the initial damage to the cell during senescence could be the direct result of the release of hydrolytic enzymes. As there was an ordered sequence of events during the early stages of senescence, it was thought that the wholesale release of enzymes was associated only with the later more degenerative processes. The enzymes that Matile claimed to be present in the vacuole, when released after the rupture of the tonoplast, would lead to changes in the cell components very similar to those actually found during the kill by paraquat and diquat. The tonoplast is normally considered to be mechanically stronger than the plasmlemma (Frey-Wyssling, 1965) but the electron micrographs of the paraquat treated cells showed rupture of the tonoplast at an earlier stage than the rupture of the plasmalemma.

It is suggested, therefore, that the actual destruction of the cotyledon leaf tissue was probably a result of the hydrolytic enzymes released from the vacuole; the possible causes of the rupture of the tonoplast will now be considered.

In addition to the work of Davenport (1960) and Baldwin (1960) (unpublished results first quoted by Calderbank, 1964), evidence has been given, from the results of experiments with the oxygen electrode, that there was a photoproduction of hydrogen peroxide by isolated chloroplasts in the presence of

Page 119 insert in line 17 at *

..... at low H_2O_2 concentrations catalase acts as a peroxidase (Chance, 1951, in 'The Enzymes', Academic Press). Plesnicar et al. (Pl. Physiol. 42, 366-370, 1967) have demonstrated peroxidase activity associated with the microbodies .....

paraquat and diquat. The results indicate that hydrogen peroxide is produced and is also rapidly utilised without the evolution of oxygen, thus suggesting peroxidation of components of the chloroplast suspension.

A major argument against the involvement of hydrogen peroxide in this way is that a high catalase concentration is found in plant cells (Neish, 1939).
Akhavein and Linscott (1968) thought it probable that excess hydrogen peroxide, formed during the auto-oxidation of the bipyridyl compounds in living tissue, would flood the plant catalase and peroxidase enzyme systems beyond the normal capacity. It appears that in flax only a small proportion of the catalase activity is associated with the chloroplasts. A similarly low activity was found by Gregory in spinach chloroplasts (1968). Although chloroplasts do contain peroxisomes (Tolbert, Oeser, Kisaki, Hageman and Yamazaki, 1968), and these do ~~not~~ exhibit catalase activity,* and thus the reduction of hydrogen peroxide within the chloroplast could only be at the expense of a substrate oxidation. Heath and Packer (1968i), working on photoperoxidation in isolated chloroplasts, demonstrated that the chloroplasts could undergo a cyclic peroxidation initiated by light. This resulted in a destruction of chlorophyll and tri-unsaturated fatty acids of the chloroplast membranes. They suggested (1968ii) that this peroxidation resulted from an overloading of the chlorophyll collection system with energy being channelled into the destructive cyclic peroxidation.

The electron micrographs showed that the first indications of tonoplast disruption occurred where this membrane was lying close to a chloroplast. It is suggested that a toxic product could be produced within the chloroplast by the interaction of photosynthetic electron transport and the bipyridyl and this diffused from the chloroplast to affect the cell membranes.

Gage (1968), working on the interaction of diquat and paraquat with mitochondrial systems of rat liver cells, suggested that these compounds were unable to penetrate the mitochondrial membrane, and conluded that it seemed more likely that transient free radicals were involved rather than hydrogen peroxide. Consequently Gage has very tentatively suggested that if plant kill is similar to the action of paraquat with mammalian cells, it might be that the paraquat does not penetrate the chloroplast but that during photosynthesis a reducing potential diffuses out which reduces the paraquat in the cytoplasm. However, it is difficult to imagine that a reducing potential would be present and able to diffuse from the chloroplasts since these are the sites of photosynthetic oxygen evolution.

Thus, in conclusion of this section of the discussion, three main features were determined during the sequence of events of the action of the herbicides diquat and paraquat. The primary direct effect of these bipyridyls was in the interruption of

normal photosynthetic electron flow with the production of some toxic substance(s) which probably included hydrogen peroxide. These brought about oxidation of cell membrane components with a consequent loss of compartmentalisation and the probable release of hydrolytic enzymes from the vacuole. The loss of membrane integrity would have upset the osmotic balance of the cell and resulted in further damage. The third and least direct effect of the action of paraquat and diquat was probably in upsetting the normal photosynthetic electron flow which would cause an overloading of the chlorophyll with a consequent bleaching and production of further hydrogen peroxide in a manner similar to that demonstrated by Heath and Packer (1968).

The interaction of bipyridyls morfamquat, diquat, benzyl viologen, paraquat and triquat with photosynthetic electron transport is now discussed.

Zweig, Shavit and Avron (1965) demonstrated that the interaction of diquat with photosynthetic electron transport was probably at a point between Photosystem I and ferredoxin. Kok, Rurainski and Owens (1965), using a method that gave a reasonable assurance of oxygen exclusion from the reaction mixture, followed the time course of photoreduction of a range of bipyridyl compounds.

They found that both the initial rate of reduction and the accumulation of reduced radicals increased with increasing concentration of the bipyridyls. However they also found that the compounds with lower (i.e. more negative) redox potentials were harder to reduce than those with higher potentials. Black (1966) found similar difficulty in reducing the more negative compounds.

The greater photoreduction of the more positive bipyridyls reported in the results and the greater mediation of oxygen uptake cannot, however, be explained by the lower energy needed for these reductions, as following a quantum theory, quantitative reductions of these bipyridyls should be the same. The possibility remains, however, that any small residual amounts of oxygen within the reaction mixtures could reoxidise the reduced bipyridyl radicals, the more negative having a far greater affinity for oxygen than the more positive. In future experiments this might be overcome by a period of illumination to remove residual oxygen before the comparative quantitative measurements are made. Kok, Rurainski and Owens (1965) commented that they obtained lower rates of reduction and accumulation of reduced radicals using CMU and ascorbate than by using glucose / glucose oxidase to trap Photosystem II oxygen evolution. The lower rates with CMU were probably due to an interfering back reaction with oxidants other than oxygen. Because of the uncertainties concerning the quantitative analysis

of bipyridyl reduction, no new evidence was obtained with regard to the normal potential of the primary reductant of Photosystem I.

The inhibition of NADP photoreduction by the five bipyridyls was found to be dependent on both the concentration of these compounds and of the chlorophyll. There was no apparent relationship between inhibition and bipyridyl redox potential. A similar chlorophyll/bipyridyl concentration effect was found with the bipyridyl mediated oxygen uptake, although at higher bipyridyl concentrations there was an increasing oxygen uptake with positive E_o', probably because they were more easily reduced. It is possible that the relationship between oxygen uptake and E_o' only occurred after saturation of the photosynthetic 'units' with the bipyridyl, a result which would account for the complete inhibition of NADP photoreduction at similar bipyridyl concentrations. In these experiments the ratio of chlorophyll to bipyridyl was found to be 150-200 to 1.

The figure of 150-200 to 1 is close to the figure reported by Baldwin, Clarke and Wilson (1968) for the amount of paraquat associated with chloroplasts isolated from sugar beet leaves which had been treated with just sufficient paraquat to kill the plant. Park and Biggins (1964) reported that 230 chlorophyll molecules were associated in a photosynthetic unit

(quantosome), but physiological methods give estimates of the size of the unit which vary with the method used for investigation. Emmerson and Arnold (1932) reported 2500 chlorophyll molecules per site of CO_2 fixation, and Izawa and Good (1965) reported a similar sized unit from DCMU inhibition studies. However, Thomas, Blaauw and Duysens (1955), measuring Hill reaction rates with broken chloroplast fragments, found that maximum rates were only lost when the size of the units was reduced to below 40-120 chlorophyll molecules.

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'Proc. 9th Brit. Weed Control Conf. 1968'

RECENT ADVANCES IN STUDIES OF THE MODE OF ACTION OF THE BIPYRIDYLIUM HERBICIDES

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Summary The mechanisms whereby diquat and paraquat are reduced in photosynthetic tissues of plants to give rise to their stable free radicals are reviewed. The reoxidation of the bipyridylium free radicals gives rise to hydrogen peroxide. Flax cotyledons damaged by the herbicides show many symptoms which occur more slowly in senescence. The chlorophyll A/B ratio is reduced, Photosystem II is lost before Photosystem I and we have demonstrated an oxidation of unsaturated lipids in the cell. Preliminary results of an electron microscopic study of damaged tissues are presented. Chloroplasts have been isolated from sugar beet leaves after treatment with herbicidal quantities of paraquat and when leaching of the material during preparation is prevented the amount of paraquat present is in the proportion of 1 molecule to 100-200 chlorophyll molecules. This ratio is related to the size of the basic photosynthetic unit.

INTRODUCTION

Aspects of the mode of action of the bipyridylium herbicides paraquat and diquat were last reviewed at this conference by Calderbank in 1964. Since this time more evidence has accumulated indicating that the herbicides can be reduced in photosynthetic systems and their competitive inhibition of NADP reduction indicates that they interact with Photosystem I (Zweig 1965, Davenport 1963). This position accords with the redox potentials of the herbicides (diquat -349 mV and paraquat -446 mV).

The reduction of these compounds gives intensely coloured free radicals which are stable in the absence of oxidising conditions. In the presence of oxygen however, a rapid reaction ensues and hydrogen peroxide is formed. No intermediate radicals can be detected in this reoxidation (Farrington 1968). In fact hydrogen peroxide has been detected in chloroplast preparations after

photoreactions involving paraquat and diquat (see Calderbank 1968). Mees (1960) showed that oxygen is necessary for herbicidal activity, indicating that the reoxidation stage rather than free radical formation gives the phytotoxic product.

We have attempted in this work to examine the sequence in which the phytotoxic symptoms appear and to study the damage which takes place in the chloroplasts of treated plant material. In some ways this is similar to what happens during senescence, though the herbicidal action is very rapid in comparison. In addition experiments are described where attempts have been made to measure the quantity of paraquat which reaches the chloroplasts of plants following a herbicidal treatment. The amount found can be related to the size of the "photosynthetic unit".

METHOD AND MATERIALS

The work relating the effects of diquat and paraquat on pigment levels, photosynthetic electron transport and membrane breakdown was carried out using flax (Linum usitatissimum var. Red Wing) cotyledons floated upon 10^{-4} M solutions of the herbicide under constant illumination. Chlorophyll and phaeophytin levels were measured spectrophotometrically in acetone solution according to MacKinney (1941) and Vernon (1960). The isolation of chloroplasts for photosynthetic electron transport studies was carried out by a method based on that of Arnon et al. (1956).

Reduction of ferricyanide was measured to indicate the activity of the Hill reaction (Photosystem II) according to Jagendorf and Margulies (1960) and the activity of Photosystem I was determined by ascorbate oxidation with the addition of monuron to inhibit Photosystem II (Davenport and Dodge).

Lipid peroxidation was followed by measuring the formation of malondialdehyde, a breakdown product of polyunsaturated fatty acid hydroperoxides, by the 2-thiobarbituric acid reaction (Wilbur et al. 1949, Heath and Packer,1965).

Sugar beet (Beta vulgaris var. Suttons Improved) were used in the measurement of quantities of herbicide reaching the chloroplasts, after a treatment just sufficient to kill (Baldwin et al. 1968). The plants were grown on a 16 hour day at about 22°C and used when they had 6-8 mature leaves. After treatment with C^{14} paraquat dichloride solutions chloroplasts were isolated in a sucrose-glycerol density gradient (Leech 1963), by the polyethylene glycol method of Clendenning et al. (1956) or after freeze drying the leaf material, by a heptane-carbontetrachloride density gradient (Stocking 1959). The amount of radioactivity in the chloroplasts prepared by these methods was assayed by liquid scintillation counting after combustion of the pellet by the oxygen flask method.

RESULTS

After treatment with the herbicides flax cotyledons change from bright green to a darker green dull appearance, which ultimately changes to brown as the plant disintegrates. Cotyledons floated upon a solution of herbicide do not desiccate in the usual way. When the pigments are examined it is seen that there is a loss of chlorophyll A, which is converted to phaeophytin A, and that this leads to a reduction in the chlorophyll A/B ratio. Chlorophyll B is affected only slightly (Table 1).

Table 1.

Changes in chlorophyll levels after paraquat treatment of flax cotyledons

Time Hours	chlorophyll content mg. per g. fresh weight 0	14	24	36
Chlorophyll A	1·40	1·25	1·05	0·5
Chlorophyll B	0·5	0·5	0·5	0·3
Ratio chlorophyll A/B	2·8	2·5	2·1	1·7

The rate of change of the chlorophyll A/B ratio under conditions of senescence, when the cotyledons were detached and floated upon water in the same way as the herbicide treatments were made is shown in Table 2.

Table 2.

Change in ratio of chlorophyll A/B during senescence of detached flax cotyledons

Time days	0	7	11	15	20
Ratio chorophyll A/B	2·7	2·6	2·1	1·9	1·7

Chloroplast preparations from cotyledons treated with diquat and paraquat for various periods showed that the Hill reaction (Photosystem II) was more sensitive than Photosystem I, and under the experimental conditions ceased completely within 24 hours, as shown in Table 3. The time of cessation of the Hill reaction is a function of temperature and Table 4 shows that damage is slower at lower temperatures.

Table 3.

Changes in photochemical activity of chloroplasts isolated from paraquat treated cotyledons

		Percentage of initial rate							
Time hours		6	12	16	18	19	24	30	36
Ferricyanide reduction	PS II	86	60	35	12	0	-	-	-
Ascorbate photoxidation	PS I	75	54	-	41	-	26	15	0

Table 4.

The effect of temperature on the loss of ability to photoreduce ferricyanide after paraquat treatment

Temperature °C	Time for complete loss of activity hours
10-12	30
16-17	28
20-21	24
23-25	19

Table 5 shows the peroxidation of the lipids of detached cotyledons after a paraquat treatment, as shown by the amount of malondialdehyde formed. It can be seen that there is a rapid increase.

Table 5.

Increase in lipid peroxidation during paraquat treatment of detached flax cotyledons

Time hours	Malondialdehyde level (percentage increase)
6	8
12	12
18	22
30	56
36	80
42	130

The amount of paraquat which reaches the chloroplasts of sugar beet leaves after a herbicidal treatment is shown in Table 6. The influence of the isolation medium is clearly seen.

Table 6.

The amount of paraquat in the chloroplast fraction of sugar beet leaves

Isolation medium	% paraquat in chloroplasts	Molecular ratio $\frac{\text{chlorophyll}}{\text{paraquat}}$
sucrose buffer	5·6	1500
	5.3	2800
	2.5	4100
	4·5	2000
Polyethylene glycol 4000	14·0	1350
Heptane - CCl_4	10·5	175
	18·9	100

DISCUSSION

Photosynthetic tissue is rapidly affected by the bipyridylium herbicides. Mees (1960) gives data indicating that broad bean leaf discs show a depression of respiration after only 3 hours of diquat treatment, and the discs form melanin and blacken more rapidly. Flax responded more slowly (Mees, 1959). Many of the changes that we have recorded here are seen in a few hours after treatment.

There is a similarity with the changes taking place in senescent tissues, where chlorophyll A is more susceptible to breakdown than chlorophyll B, and the ratio chlorophyll A/B falls, but this process normally takes many days. Thus the herbicide treatment considerably increases the speed of degeneration (compare Table 1 and 2). Treatment of the cotyledons with monuron, a general photosynthetic inhibitor, reduces the speed at which the chlorophyll A is lost.

It has been noted however that the ability to photoreduce ferricyanide is lost from untreated chloroplasts very rapidly, and Photosystem I has been shown to be very stable, and will survive heat treatment and sonication (Davenport and Dodge). Although the herbicides interact with Photosystem I loss of Photosystem II activity occurred before that of Photosystem I.

The oxidation of lipids by isolated chloroplasts has been studied by Heath and Packer (1965), who concluded that it was a light dependant reaction. In the normal plant cell a protection mechanism must be present to prevent damage taking place. The lipids in the plant cell are constituents of membranes which regulate the osmotic stability of the system. Thus the changes in malondialdehyde that we have shown indicate that after paraquat and diquat treatment a rapid upset of membrane integrity is possible, and this may well explain the speed at which symptoms of damage can be seen.

We have recently obtained electron micrographs of cotyledons treated with the bipyridylium herbicides and these show shrinkage of the cytoplasm from the cell wall and gross enlargement of the mitochondria, as well as the production of osmiophilic granules or plastoglobuli (Lichtenthaler,1968) in the chloroplasts. The formation of these granules again demonstrates the similarity between the damage caused by the herbicides and that which takes place in senescence. Many of the effects described may be secondary to the reaction which damages the leaf tissue cells. Once damage, perhaps to a membrane, is caused the breakdown of the tissue follows the normal pattern seen in ageing and dying cells. The relationship between hydrogen peroxide and damage has still to be elucidated, and the reason why catalase, which is present in plant cells, does not prevent damage by destroying the peroxide is intriguing. Unpublished results show that catalase in photosynthetic tissue is present to only a small extent in the chloroplast, and this is confirmed by Gregory (1968).

Table 6 indicates that paraquat can be leached out of chloroplasts by aqueous solutions, and in fact 90% can be removed in three aqueous washings. Up to 20% of the paraquat used to treat a leaf reaches the chloroplast, and this indicates that there are between 100-200 molecules of chlorophyll present to every herbicide molecule. Molecules of chlorophyll are known to work together as aggregates, though estimates of the size of this basic unit, or quantasome, vary from between 230 and 2,500 chlorophyll molecules (Park and Biggins, 1964; Izawa and Good, 1965). Whichever figure is taken it appears that there is more than enough herbicide to interact with each quantosome. Furthermore these results confirm that the proposed mechanism of photosynthetic reduction of the herbicide is possible, for paraquat does reach the chloroplast.

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The Mode of Action of Paraquat and Diquat

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Paraquat (1,1′-dimethyl-4,4′-bipyridylium dichloride) and diquat (1,1′-ethylene-2,2′-bipyridylium dibromide) are widely used in formulations as quick acting desiccant herbicides. They are the active components of the herbicides Reglone and Gramoxone, marketed by Plant Protection Ltd., Bracknell, Berks., U.K. Initial work on the mode of action of diquat by Mees (1960) indicated that oxygen and light were essential for the kill of green plants. Zweig, Shavit & Avron (1965) further elucidated the light requirement in experiments with isolated chloroplasts. They found that diquat caused a deviation of electron flow from photosystem I, leading to an inhibition of $NADP^+$ reduction and the production of a reduced diquat radical. A possible requirement for oxygen was established by Davenport (1963), who found that the reduced diquat free radical was reoxidized with the production of hydrogen peroxide.

Further evidence for the production of hydrogen peroxide has been obtained in experiments with heated chloroplasts, which have no photosystem II activity, and no requirement for dichlorophenolindophenol (Davenport & Dodge, 1969) to mediate electron flow from ascorbate. In the presence of paraquat illuminated chloroplasts showed an oxygen uptake, part of which was released by the addition of catalase in the dark. However, if catalase and ethanol were included in the chamber during illumination, acting as a trap for hydrogen peroxide, no oxygen evolution was obtained on the addition of further catalase.

By using flax (*Linum usitatissimum* var. Red Wing) cotyledon leaves floated on a 0.1mM solution of paraquat or diquat under continuous illumination, the sequence of some of the events occurring during the kill was followed.

Photosystem II activity, as measured by ferricyanide reduction of isolated chloroplasts, was inoperative after 20h of treatment in the light. At this stage there was little visible evidence of herbicidal activity. As photosystem I, with which the bipyridyls interact, is dependent on photosystem II *in vivo*, we concluded that there was no further production of primary toxic products after 20h of continuous illumination. When leaves were illuminated for 6h and then put into the dark, photosystem II inactivation continued, but at a lower rate. If a dark period was commenced after 15h of continuous illumination, the light and dark inactivations from this point were identical. With chlorophyll contents, however, a lower rate of breakdown was observed in the dark, even after 20h of continuous illumination. This increased breakdown was probably due to photo-oxidation. The breakdown of chlorophyll increased rapidly in continuous illumination after 20h, with a more rapid breakdown of chlorophyll *a* than of chlorophyll *b*.

Malondialdehyde, a breakdown product of polyunsaturated fatty acid hydroperoxides (Kohn & Liversedge 1944; Patton & Kurtz, 1951), was measured in treated leaves as an indication of lipid peroxidation. Production began after 6h of herbicide treatment, and continued to increase during the next 48h. The loss of galactosyl lipids indicated that there could well be a breakdown of cell membranes, and electron micrographs showed a disruption of the tonoplast in some cells after 6h. There was an increase in membrane permeability at this time, as indicated by an increased efflux of K^+ from treated leaves (cf. Eilam, 1965). Soluble protein contents were shown to decrease. Electron micrographs obtained after up to 30h of treatment showed a massive increase in the number and size of osmiophilic globules (Lichtenthaler, 1968) and also a considerable change in the appearance of chloroplast nucleic acids.

It is considered that the breaking of the tonoplast would lead to a rapid change in osmotic equilibrium, which would have considerable effects on the cytoplasmic organelles. If the vacuole is classed as a lysosome (Matile & Moor, 1968), the release of hydrolytic enzymes, including proteases, nucleases and phosphatases, would have a dramatic effect on cell components.

Although the pattern of breakdown of many cell consituents is similar to that found during natural senescence but on a shorter time-scale, significant differences in comparative times of changes in membrane permeability and photosynthetic activity (Baldwin, Dodge & Harris, 1968; Drury & Park, 1968) indicate that the herbicidal activity is a fundamentally different process.

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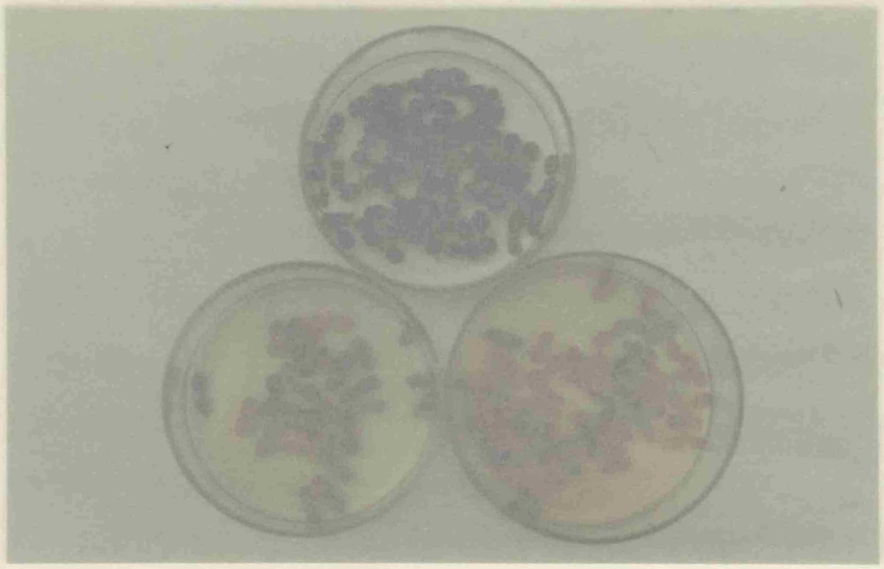
Plate 1.



control

60 hours paraquat

90 hours paraquat

Figure 15. Chromatography of total lipid extracts of control and 40 hour paraquat treated flax cotyledon leaves.

A sprayed with phosphomolybdic acid

B sprayed with periodate-Schiff reagents

C sprayed with molybdophosphoric acid

a pigments

b mono and diglycerides

c monogalactosyl diglyceride

c' " " from digalactosyl diglyceride

d phosphotidyl ethanolamine

e phosphotidyl glycerol

f digalactosyl diglyceride

g phosphotidyl choline